T113
+Y12
3327
YALE UNIVERSITY LIBRARY
3 9002 06584 8351

MEASUREMENT OF SYMPATHETIC NERVOUS SYSTEM ACTIVITY IN THE RAT KIDNEY

John Francis McQuade III

1973

MUDD
LIBRARY
Medical

YALE

MEDICAL LIBRARY

Permission for photocopying or microfilming of "Measurement of Sympathetic Nervous System Activity in the Kidney"

(TITLE OF THESIS)

for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

John F. McDonald III

Signature of Author

5/8/73

Date

Digitized by the Internet Archive
in 2017 with funding from
Arcadia Fund

https://archive.org/details/measurementofsym00mcqu

MEASUREMENT OF SYMPATHETIC NERVOUS SYSTEM ACTIVITY IN THE RAT KIDNEY

John Francis McQuade III

B.S. Yale University

This thesis is presented in partial fulfillment of the requirements for the degree of Doctor of Medicine

Department of Medicine

Yale University 1973

I dedicate this thesis to

my wife, Diane, and to my parents

ACKNOWLEDGEMENTS

Throughout the course of a medical education, one encounters a few outstanding individuals who have a profound influence on one's maturation. I have been fortunate in having known three such people during my four years at Yale Medical School.

It is impossible to fully express my deepest gratitude to Dr. Lewis Landsberg for his friendship and his role as my thesis advisor. Aside from providing valuable suggestions vis-à-vis experimental design, his support and encouragement continually reinforced my enthusiasm for this research effort. In addition to being a clinician and teacher of the highest caliber, Lewis is a truly fine human being.

I also wish to express my sincerest appreciation to Dr. Mark Hayes for his wise counsel and warm friendship. He is a rarity in the professional world of medicine today because of his combined excellence in the fields of general surgery and internal medicine. Moreover, Dr. Hayes has never failed to treat his patients with the respect and compassion they all deserve. It is to these qualities that I shall devote my professional life.

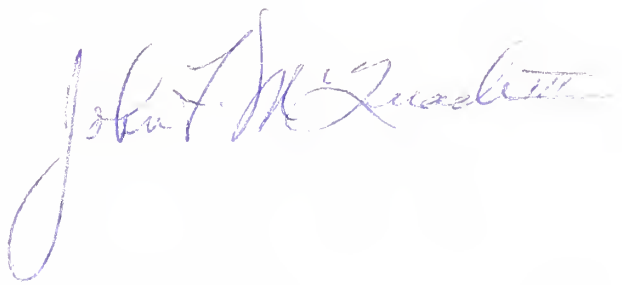
Finally, I'd like to acknowledge Dr. Marie Browne as the finest teacher of clinical medicine at Yale. Without a doubt, those six weeks in her Pediatric Cardiology Clinic represented the most satisfying and exciting clinical experience of my entire undergraduate medical

education. Her qualities as a teacher and clinician are only equaled by her warmness and honesty as an individual.

Special thanks to Dr. Robert Levine, who freely devoted much time and expertise in assisting with the writing of this thesis. I am grateful to Dr. Howard Taubin whose comments, funds, and encouragement also contributed to this effort. Mrs. Susan Bruno, Dr. Landsberg's laboratory assistant was frequently a life-saver. Her help with the column chromatography is truly appreciated. Only those who have labored over these columns can be fully aware of what such assistance means. Thanks, Susan.

Thank you Emanuel Lerner for your time, effort, and good nature, vis-à-vis the statistical analysis. An excellent statistician, "Many," as we affectionately refer to him, is one hell of a great guy.

Finally, I must express my appreciation to Mrs. LaVerne Kimball for her very fine typographical assistance. I've known LaVerne for three years while on the Yale-New Haven Blood Bank staff, and count myself a better individual for having this opportunity.



The maintenance of extracellular fluid volume within a narrow range is an essential prerequisite for establishing Claude Bernard's "constancy of internal milieu" within the human organism. As a predominant solute of ECF, sodium salts represent the major determinant of this volume. Hence, an appreciation of the metabolism of sodium under normal physiologic circumstances is of utmost importance in understanding normal sodium homeostasis as well as the pathophysiology of the various salt-retaining, or edematous, states.

Aside from the absorption or loss of sodium by way of the gastrointestinal tract or skin, the kidney represents the major site of sodium conservation. Here, as a consequence of relatively subtle changes in extracellular volume (under normal conditions), sodium is either reabsorbed or excreted in an attempt to preserve the normal volume of this fluid compartment. The amount of sodium excreted depends on the difference between the amount filtered by the glomeruli, and that reabsorbed by the tubules. Clearly, alteration of either parameter will have pronounced effects on the quantity of sodium excreted in the urine. This relation has been known for several decades. However, what remains obscure at the present time are the following: (1) The manner in which the kidney perceives subtle changes in extracellular fluid volume and (2) the translation of these signals into various renal mechanisms operative in altering sodium excretion.

Prior to 1961, it was generally felt that control over sodium excretion by the kidney was the resultant of two major events or "factors": glomerular filtration and the sodium reabsorptive effect of aldosterone. It was recognized then, as now, however, that mere alterations in GFR do

not bring about proportional changes in sodium excretion. At the proximal tubular level, the phenomenon of glomerulotubular balance is operative. Nonetheless, the rate of glomerular filtration can play a major role in the absolute excretion of sodium for a given rate of tubular reabsorption [1]. In addition, the ability of aldosterone to facilitate sodium reabsorption at the distal tubular level has long been acknowledged.

De Wardner [2] demonstrated in 1961, however, that the above two factors did not operate alone in controlling sodium metabolism. During an infusion of saline in mineralocorticoid-treated dogs, the abdominal aorta was constricted above the take-off of the renal arteries, thereby diminishing renal blood flow and glomerular filtration. Unexpectedly, sodium excretion persisted despite a decrease in GFR and high levels of DOCA. These results were confirmed in several laboratories. The conclusion to be drawn was that other non-aldersterone factors influenced the tubular reabsorption of sodium. Several of these factors have been subsequently elucidated [1,3,4]. They included: peritubular oncotic and hydrostatic pressure; intrarenal redistribution of blood flow to nephron populations of differing reabsorptive capacities; and a postulated natriuretic hormone. Together, these hormonal and hemodynamic variables influencing sodium reabsorption encompass what the literature has referred to as the "third factor."

A discussion of filtration fraction serves to illustrate the importance of "third factor." By definition, this is a ratio of GFR to RBF, and may be regarded as an index of the relation between glomerular filtration and post glomerular hydrostatic pressure. With saline infusion, there is a decrease in renal vascular resistance and a concomitant decrease in filtration fraction (FF). As a consequence, peritubular hydrostatic

pressure is elevated, and sodium reabsorption (luminal to peritubular) depressed. Conversely, in a hypovolemic situation, if perfusion pressure falls--but to a degree not sufficient to decrease GFR--the FF will rise. Peritubular hydrostatic pressure declines and sodium reabsorption is enhanced.

The importance of these peritubular forces has recently been emphasized in a paper by Giebish [5]. Indeed, the phenomenon of glomerulotubular balance is in all probability a resultant of these intrarenal, third factor mechanisms. But as mentioned previously, the manner in which the kidney perceives changes in ECF volume and translates such information into alterations of GFR, aldosterone secretion, and third factor--so as to effect changes in sodium reabsorption--remains undefined. However, much evidence has been adduced implicating a significant role for the sympathetic nervous system in this process. Herein lies the stimulus for the undertaking of this thesis.

The remainder of the introduction will be devoted to demonstrating the role played by the sympathetic nervous activity in renal sodium metabolism. In so doing, a firm experimental-theoretical foundation will hopefully be constructed, from which the justification for my thesis and conclusions drawn therefrom should be evident.

From a gross anatomic standpoint, the kidney receives autonomic postganglionic fibers from the celiac and superior mesenteric ganglia. Preganglionic fibers are derived from the splanchnic nerves. At a finer level, various workers have described sympathetic innervation to afferent and efferent arterioles, venules, and vasa recta [6,7,8].

A study performed by Ljungqvist [9], comparing rat to human

neurovasculature, revealed the importance of correlating renal innervation and vascular anatomy with function. In both species cortical arteriolar continuity is interrupted by glomerular tufts. In fact, the tufts constitute the only connection between afferent and efferent vessels. (Efferent arterioles later subdivide to form a peritubular capillary plexus.) In contrast to this, arterioles within the juxtamedullary zone are continuous vessels leading to the medulla. Here, glomerular tufts are elaborated as side branches of this vessel; and afferent and efferent arterioles are in anatomic continuity at the vascular pedicle of the tuft. In this study, Ljungqvist demonstrated, by fluorescence microscopy, adrenergic innervations along afferent and efferent vessels at both cortical and juxtamedullary levels.

The physiologic consequence of this vascular architecture with its autonomic associations are evident [9]: Increased adrenergic activity at the juxtamedullary level with resulting vasoconstriction here, should reduce the capacity of these extraglomerular medullary pathways and shunt renal blood flow to the cortex. Here, no extraglomerular routes are available and GFR would increase, thereby augmenting filtration fraction and net sodium reabsorption (third factor effects).

Much evidence has been advanced to suggest an active "in vivo" role of the sympathetic nervous system in the control of renal vasomotion and sodium balance. Zimmerman et al. [10] stimulated the renal nerves of healthy mongrel dogs. This produces marked vasoconstriction and a concomitant release of catecholamine (norepinephrine) in the venous effluent. Concha [11] cannulated the artery and vein of the left kidney of a dog, leaving the renal nerves intact. (Perfusate was Tyrode's solution.) He noted that renal vasoconstriction was consistantly evoked by stimulation

of the left major splanchnic nerve. No vasomotion was produced by excitation of the subdiaphragmatic vagi. In another interesting experiment, he collected the perfusate following nervous stimulation and re-injected it into the kidney. Vasoconstriction was subsequently elicited. No such effect was demonstrated with perfusate from a non-stimulated kidney or saline control. These results suggested that vasoconstriction was associated with the release of a vasoconstrictor substance following nervous stimulation. Direct infusion of norepinephrine (0.0001 μg/ml) provided strong vasoconstriction. Finally, the ability of Hydergine (dihydroergotoxine mesylate), and α-adrenergic antagonist, to block the vasoconstrictive response to renal nerve stimulation, suggested the adrenergic nature of renal innervation. All these results are consistent with the hypothesis that renal innervation is chiefly adrenergic, and that sympathetic activity plays a role in vasomotion here.

Clearly, sympathetic nervous input to the kidney does not operate in a vacuum. On the contrary, it is one manifestation of overall sympathetic discharge resulting from such stimuli as hypovolemia, excitement, hypoglycemia, etc.; and it participates in concert with other sympathetically innervated structures to restore the organism to a state of equilibrium. The relationship of renal nerve activity to hypothalamic activity and the baroreceptor reflex--both preeminent modulators of sympathetic activity--serves to illustrate this point. Having performed bilateral carotid occlusion on dogs, Iriuchijima [12] demonstrated an increase in mean renal nerve activity. Similar results are obtained by sectioning of baroreceptor afferent nerves [13]. In a study on lightly anesthetized cats, Wilson et al. portrayed mean renal nerve activity as a "linear combination of hypothalamic, basal central neural (medullary or supramedullary), and

baroreceptor reflex activity" [14].

A more clinically related experiment--and perhaps more provocative--was performed by Gill and Casper [15], when they investigated the role of the sympathetic nervous system in the renal response to hemorrhage. Hemorrhage, of course, calls forth an increase in sympathetic activity secondary to the baroreceptor reflex. And, as demonstrated above, this adrenergic activity is transmitted to the kidneys and manifested as alterations in RBF, GFR and sodium excretion. In this experiment a DOCA-treated canine donor was used to perfuse (by cross-circulation techniques) the kidney of a DOCA-treated recipient (dog) whose renal nerve supply was left intact. At all times throughout the experiment, the mean arterial pressure in donor and recipient kidneys was constant and equal. A natriuresis was induced in both donor and recipient kidneys by saline-albumin infusion in the donor. Then, the recipient animal was bled so as to elicit marked sympathetic activity to the perfused kidney--which was otherwise protected from the other changes accompanying hypovolemia. The results were striking: sodium excretion in the perfused recipient kidney was reduced by 50 per cent, without significant change in sodium output of the donor. This diminution in sodium excretion occurred despite continued exposure of the perfused kidney to such natriuretic stimuli (arising from the donor circulation) as hemodilution, natriuretic hormone, etc.--all of which would otherwise oppose sodium conservation. Furthermore, when the sympathetic nerve supply to the perfused kidney was interrupted, there was no change in sodium excretion in response to hemorrhage--even with a 42 per cent decrease in mean arterial pressure in the recipient. Hence we are provided with "prima facie" evidence that the sympathetic nervous system does play a role in renal sodium metabolism, at least under

hypovolemic circumstances.

Several other studies have implicated the adrenergic nervous system in renal sodium metabolism. In man, it has been shown that sympathetic blockade with guanethidine impaired sodium conservation during periods of sodium deprivation [16]. There have been several case reports of impaired ability to reabsorb sodium in patients with insufficiency of the autonomic nervous system [17]. Finally, it has been pointed out that adrenergic blockade facilitates sodium excretion in normal subjects administered saline infusion or treated with mineralocorticoids [18].

The actual mechanism(s) by which sympathetic nervous activity effects alterations in sodium balance has only been alluded to so far, vis-a-vis Ljungqvist's correlation of renal vascular and neural architecture. In 1947, Trueta, et al. [19], provided some insight into this problem by demonstrating reductions in cortical blood flow--with maintenance of medullary flow--during periods of shock in dogs. Since these observations, much effort has been directed toward ascribing an active role for the sympathetic nervous system in the intrarenal redistribution of blood flow. More clinically related research topics have included the association of this phenomenon with hypovolemia, sodium deprivation, or edema forming states. Before citing some of the work accomplished in this area, a consideration of the physiologic consequences of intrarenal flow redistribution and how it relates to third factor would be appropriate. From basic physiology one may recall that four renal medullary factors regulate renal concentrating--and sodium reabsorbing--ability: (1) the rate of sodium pumping and countercurrent multiplication by the loops of Henle, (2) the rate of washout and dilution of medullary solutes by medullary blood flow, (3) the rate of washout and dilution of medullary

solutes by urine, and (4) the permeability of collecting duct eipthelium [20]. Compared with countercurrent exchange in the cortex and outer medulla, that occurring in the inner medulla via long parallel vasa recta is exceedingly more efficient. Moreover, in the outer medulla and cortex, there is greater washout and dilution of sodium and urea, leading to decreased concentrating ability and sodium reabsorption. Augmentation of medullary blood flow presumably lowers sodium concentration in the medulla, thus enhancing reabsorption by the loops of Henle (2° to smaller back diffusion of sodium). In addition, arteriolar vasoconstriction in the outer cortex decreases hydrosatic pressure in the peritubular capillaries, thereby encouraging proximal reabsorption of sodium and water. Finally, salt reabsorption is enhanced at the medullary level by virtue of the longer loops of Henle located there. Hence, if blood is shunted from the cortex to the medulla--as may be the result of cortical vasoconstrictive sympathetically-mediated activity--sodium reabsorption would be promoted.

Pomeranz [20] studied the effect of renal nerve activation on intra-renal capillary blood flow in conscious and anesthetized dogs. This was accomplished by eliciting a chronic or acute carotid sinus reflex and by chronically or acutely stimulating the splanchnic nerve. Techniques employed here included 85 Kr radioangiography, 85 Kr clearance studies monitored by x-ray scintillation and preparation of silicone rubber casts of the microvasculature. The conclusions drawn from his experiments were that mild renal nerve stimulation--which may not alter overall RFB--does result in diminished perfusion of the outer cortical peritubular capillaries. Concurrently, perfusion appears to be increased in the outer medulla. Furthermore, such redistribution in chronically stimulated animals could be reversed with infusions of phenoxybenzamine into the renal artery or

blockage of nerve conduction with xylocane.

The clinical implications of such an experiment are evident. As a result of sodium depletion or hemorrhage, intravascular volume contracts and the blood pressure falls. Activation of baroreceptor and central vasomotor autonomic reflexes would then lead to renal nerve stimulation with concomitant redistribution of blood flow in the kidneys (cortex to medulla). Sodium and water is reabsorbed with greater efficiency, and the blood pressure is ultimately reestablished. Carriere [21] provided further evidence in support of this reasoning by investigating the intrarenal distribution of blood flow in dogs during hemorrhagic hypotension. Employing techniques similar to those mentioned above [20], he showed that such hypovolemia led to progressive diminution of cortical flow; while flow rate within the juxtamedullary compartment was well maintained. Since the flow rate was normal at half the blood pressure in this latter region, vascular resistence here must have been reduced by 50 per cent. Conversely, vascular resistance must have been elevated 100 per cent in the outer cortex. Involvement of adrenergic activity was suggested by this worker, in view of similar patterns of redistribution resulting from renal nerve stimulation, or renal arterial infusions of catecholamines.

During states of sodium deprivation in rats [22] and man [23] preferential cortical to medullary shunting of RBF has been demonstrated. The consequences of this with respect to sodium metabolism are of major importance, particularly when the animal needs to retain sodium--or does so obligatorily--whether under conditions of a low sodium diet or under the stimulus of an edema forming state. As stressed before, the longer intramedullary nephrons are more efficient at sodium reabsorption. Moreover, a decreased cortical single nephron glomerular filtration rate

(SNGFR) (as was observed in rats during low sodium diets [22]) should allow for more thorough sodium reabsorption in these shorter nephrons. Indeed, one need not even postulate a decreased SNGFR within the cortex; since mere reduction of RBF (via sympathetically mediated arteriolar vasoconstriction with maintenance of GFR will increase the filtration fraction. Peritubular hydrostatic pressure falls as a result, oncotic pressure rises, and sodium reabsorption is facilitated via these third factor forces.

Perhaps the most exciting investigations concerning the interaction of the sympathetic nervous system and renal mechanisms of sodium metabolism have focused on the relationship between adrenergic activity and release of renin by the kidney. Anatomic studies have demonstrated fluorescent varicosities, having the same morphologic characteristics as norepinephrine-containing nerve terminals, in the walls of intrarenal arterioles [24]. Moreover, this innervation has been found to extend into the parts of these arteriolar walls containing granulated juxtaglomerular cells--the storage sites of renin. This work, together with that of Ljungqvist [9], Barajas [25,26] and others, suggests, but does not prove, a direct role of adrenergic innervation on release of renin by the juxtaglomerular cells.

Somewhat more direct evidence in favor of this argument has derived from several more physiologic-biochemical experiments. For example, plasma renin activity (PRA) is elevated in anesthesized dogs given intravenous epinephrine or norepinephrine, or whose renal nerves have been stimulated (with maintenance of constant renal arterial pressure) [27]. Toquini et al. [28] described a decline in the renin content of rat kidneys following denervation.

In attempt to establish the existence of autonomic influence on renin

release, Bunag, Page, and McCubbin conducted a provocative experiment on anesthetized adult mongrel dogs [29]. These animals were hemorrhaged to a degree sufficient to engender diffuse vasomotor discharge (as evidenced by compensatory tachycardia etc.). This resulted in an increase in PRA, even though renal perfusion pressure was artificially held constant. Since a rough correlation existed between the compensatory tachycardia and renin release, the effect was felt to be sympathetic. Moreover, adrenergic discharge elicited by carotid occlusion also produced an elevated PRA (again with constant renal perfusion pressure). Renin release was prevented with ganglionic blockade (TEAC) or by local anesthesia of the renal nerves. In contrast, infusions of norepinephrine, tyramine, or DMPP (dimethylphenylpiperazinium, a ganglionic stimulator) evoked elevated PRA. Finally, all these results were amplified by placing the animals, beforehand, on a low salt diet.

Mogil measured PRA in normotensive and renal hypertensive dogs in the presence and absence of renal innervation, under a variety of stimuli designed to affect renin-angiotensin activity [30]. His conclusion was than intact renal sympathetic innervation is necessary for the renin-angiotensin system to respond normally to changes in sodium balance. However, it should be noted that others have reported elevated renin-angiotensin levels in denervated dogs during and following hemorrhagic shock [31]. Mogil offers two possible modalities by which the sympathetic nervous system might affect the release of renin: (1) by altering afferent arteriolar vasoconstriction proximal to (or at the site of) the juxtaglomerular baroreceptor--thus changing local hemodynamics and subsequent release of renin, or (2) by increasing the sensitivity of juxtaglomerular cells to various stimuli which affect the release of renin.

From a more clinical standpoint, measurements of PRA have been made in man, following intravenous infusion of catecholamines and in rats, in response to cold stimulation of the sympathetic nervous system [32]. Despite concurrent elevations of blood pressure, PRA and aldosterone were observed to be increased. Similarly, sodium depletion and upright posture resulted in rising PRA and urinary catecholamines and aldosterone. However, patients with severe autonomic dysfunction, who were salt depleted or in upright posture, did not experience an elevation of any of these values--notwithstanding a substantial fall in BP. But when their orthostatic hypotension was prevented by iv. catecholamines, there was noted to be a normal rise in PRA and aldosterone in response to the previously mentioned challenges. Further studies such as this support the contention that the sympathetic nervous system does play a direct intrarenal role in the mediation of the renin-angiotensin system [33,34].

Such observations and arguments, as presented above, permit a more physiologic approach toward comprehending the pathophysiology of various salt-retaining or edematous states. For example, greater than normal amounts of catecholamines have been found in the urine of nephrotic patients, suggesting increased sympathetic activity probably secondary to a contracted circulating blood volume [35]. Concomitantly, renin and aldosterone are elevated. In congestive heart failure, catecholamine levels are characteristically increased in both urine [36] and plasma [37]; several observers have described greater than average arterial and venous tone [38]. As in nephrosis, this apparent heightened sympathetic activity probably reflects a relative (or absolute) hypovolemia. Its major intrarenal effect is probably a vasoconstrictive one altering such third factor forces as filtration fraction, redistribution of blood flow, etc., so as

to facilitate sodium reabsorption. Finally, renal vascular resistance is increased along with depressed RBF in hepatic cirrhosis, despite overall expansion of extracellular volume and cardiac output [39]. There is evidence to ascribe these changes to decreased arterial filling (via adrenergic activity), leading to higher FF and redistribution of blood (cortex to medulla). Sodium reabsorption is enhanced.

In summary, there appears to be one abnormality common to all of the above mentioned edema-forming states: an inadequacy of arterial filling--whether a consequence of contracted blood volume, decreased cardiac output, or supernormal run-off of arterial blood to venous circulation via fistula. Such a situation leads to increased adrenergic activity with the following sequelae: (1) immediate defense of the circulation by augmentation of cardiac output and peripheral arteriolar vasoconstriction, (2) secretion of renin and aldosterone, in part due to sympathetic mediation, (3) renal vasoconstriction with intrarenal flow redistribution, resulting in depressed RBF and concomitant physical hemodynamic factors contributing to increased sodium reabsorption.

An experimental model of low-output congestive heart failure has been constructed by constriction of the thoracic inferior vena cava (TIVC) in dogs. Such a maneuver effects a decline in the circulating blood volume with two major consequences. First, the arterial pressure is defended by reflex baroreceptor stimuli to the vasomotor center. Resultant efferent adrenergic stimuli augment cardiac contractility and peripheral vasoconstriction. Second, an attempt to restore intravascular volume to an effective level is made by increasing sodium reabsorption via an interplay between the determinants of GFR, aldosterone secretion, and third factor.

Kilcoyne et al. studied the effect of TIVC occlusion on intrarenal

blood flow and sodium hemostasis in mongrel dogs [40]. ^{133}Xe washout minotored by γ-ray scintillation was employed in this investigation. Paralleling the results of others [20,21,23], his data indicated that acute TIVC occlusion produces a marked sodium retention and significant redistribution of renal blood flow. This he characterized as diminished perfusion in the superficial cortex with its short nephron population and rich sympathetic innervation. Moreover, while whole kidney GFR remained constant, urinary sodium excretion declined. As a corollary to these findings, it was observed that unilateral infusion of norepinephrine produced similar vasomotor changes and renal sodium retention. In addition, he found that renal denervation or infusion of α-adrenergic blocking agents reduced, but did not prevent, TIVC occlusion-induced vascular redistribution [41]. The implied role of the sympathetic nervous system here is obvious. And the significance of these results relative to the pathogenesis of edema should be stressed; since similar redistribution of blood flow and reduced sodium excretion has been noted in dogs with experimental heart failure [42], pulmonary artery constriction, or tricuspid avulsion [39].

Summary: This review has emphasized that sodium metabolism is modulated not only by variations in overall GFR and aldosterone-mediated tubular reabsorption, but also by an amalgamation of third factor influences. These include changes in peritubular hydrostatic or oncotic pressure, a postulated natriuretic hormone, and intrarenal distribution of blood flow (or glomerular filtrate). Much evidence--anatomic, physiologic, and pharmacologic--has been adduced to establish a significant role for the sympathetic nervous system in these third factor forces. Moreover, an important interplay between renal adrenergic activity and renin secretion

has been suggested. Finally, these physiologic observations have been placed into a clinical perspective by defining the part they may play in the pathogenesis of edema.

FINE STRUCTURE OF THE SYMPATHETIC NERVOUS SYSTEM

In contrast to the distinct neuromuscular junction of motor neurons, sympathetic nerves terminate in an extensive ground plexus. Each fiber of this branching network is in close proximity to several effector cells and is characterized as having many beaded dilations, or varicosities, along its length. Within these varicosities are mitochondria and membrane bound vesicles, much akin to those described in the adrenal medulla [43].

The intraneuronal distribution of the sympathetic neurotransmitter, norepinephrine (NE), is uneven, with small concentrations in the soma and axon, but large concentrations in the varicosities. Several workers have localized the NE within varicosities to the adrenergic vesicles [44,45]; although a small extravesicular pool does exist. There is virtually no extraneuronal NE in adrenergically innervated tissue [46].

Three types of synaptic vesicles have been described in axon varicosities: (1) small agranular vesicles (300-600Å diameter) similar to those seen in most cholergic nerve endings, (2) small granular vesicles (diameter 300-600Å) with dense cores (diameter 150-250Å), and (3) large granular vesicles diameter 600-1500Å) with a central core (400-700Å). That NE is predominantly localized in small granular vesicles (hereafter often referred to as "granules"), has been concluded from electron microscopic, histochemical, and microfluorimetric investigations [46].

Through such techniques as differential and density gradient centrifugation, the biochemical characteristics and morphogenesis of the granules has been partially elucidated. They are membrane bound,

assembled in the cell body, and transported along the axon to the nerve ending. Here, their life span has been estimated to be 5-10 weeks [47]. In addition to storing NE, vesicles contain lipid, phospholipid, proteins, RNA, and ATP. The protein components, of which dopamine-β-hydroxylase is one, are referred to as chromogranins. Aside from the biosynthetic role of dopamine-β-hydroxylase, the role of chromogranins remains undefined; although much speculation focuses on their participation--along with ATP--in the intravesicular binding of NE.

The adrenergic nerve terminals contain all the enzymes, cofactors, and precursors necessary for NE synthesis and storage. Tyrosine hydroxylase, the first enzyme in the biosynthetic pathway and apparently the rate-limiting one [48], is confined to the soluble fraction of the cell. Prior to hydroxylating L-tyrosine to L-Dopa the enzyme must first be hydrogenated by a reduced pteridine cofactor. A dihydropteridine reductase is present in the adrenal medulla, and presumably, in sympathetic neurons. Also located in the soluble cell fraction is aromatic L-amino acid decarboxylase. It is a pyridoxal-requiring enzyme and catalyzes the conversion of L-Dopa to dopamine (DA).

Unlike the previously mentioned enzymes, dopamine-β-hydroxylase is within the storage granules. Indeed, DA must be taken up by the granules prior to its β-hydroxylation. There is some evidence available to suggest that most dopamine-β-hydroxylase is bound to the sympathetic neuron vesicular membrane [46].

The regulatory mechanisms of catecholamine biosynthesis are not unlike those described for other anabolic pathways. Rate of NE synthesis is a function of the availability of cofactors, enzyme substrates, and the amounts and activities of these enzymes. NE contributes to the control

of its biosynthesis via feedback inhibition of trysoine hydroxylase, probably by competition with the pteridine cofactor for the oxidized form of the enzyme. It has been postulated that the small extravesicular pool of free NE is involved in this competitive feedback inhibition. At a higher level of organization, changes in sympathetic activity are reflected by concurrent alterations in catecholamine turnover rates, though tissue levels remain constant. In fact, prolonged sympathetic nerve stimulation results in enzyme induction of tyrosine hydroxylase [47,49]. Finally, there is biosynthetic regulation at the interface of various intracellular compartments, i.e. granular uptake of dopamine may be rate-limiting under certain circumstances [47].

The uptake and release of NE by in vitro granular preparations has been investigated by several workers. Within one minute after iv. ^{3}H-NE into rabbits, Titus noted that 25 per cent labeled NE was recoverable in particle-bound form from homogenates of the heart [50]. Similar results are obtained in the rat [51]. The uptake process appears to be temperature dependent, with lowest activity at 4°C [52]. Although even at 0°C, splenic nerve vesicles are permeable to NE, permitting exchange of ^{3}H-NE with endogenous NE in vesicles [53]. Indeed, Potter [52] describes the immediate adsorption of 17 times the solution concentration (10^{-7}M) of NE to splenic nerve granules at 4°C. Owing to the heterogeneity of granular preparations, there is great variation in the inhibition of vesicular NE uptake by such agents as reserpine or EDTA [43,54,52]. Finally, it appears that NE must be stored in the granules prior to release [47]--a process much like that occurring at the neuromuscular junction.

Many studies have supported the generally accepted notion that minature endplate potentials and synaptic transmission at the skeletal

neuromuscular junction are mediated by complete presynaptic exocytosis of acetylcholine-containing vesicles. Evidence for a similar "quantal" release of NE-containing vesicles during synaptic transmission in adrenergic neurons, is not nearly as extensive as that obtained for the skeletal neuromuscular junction. However, all electrophysiologic and biochemical data gathered so far can be explained in terms of this excitation-secretion coupling model [46,52,55-59]. Strong parallels can be drawn to the secretion of catecholamines by the adrenal medulla. The mechanism by which the vesicular contents are discharged into the extracellular space, leaving the membrane behind in the cytoplasm, is not clearly understood. Evidence points to an interaction of the sympathetic nervous stimulus, calcium influx, and the resultant granular membrane effect producing vesicular exocytosis [47].

Having been released into the extracellular space, the effect of NE is short-lived; as it is rapidly taken up into the sympathetic nerve. Extracellular NE is also catabolized to O-methylated derivatives; although this is quantitatively of less significance than re-uptake. Briefly, the re-uptake mechanism is located in the axonal membrane, and quite distinct from the similar process at the granular level. Drugs such as cocaine, sympathomimetic amines, receptor antagonists, neuron blocking agents, phenothiazines and tricyclic antidepressants block re-uptake.

TURNOVER OF CATECHOLAMINE

Nearly all NE outside the adrenal medulla and CNS is located in peripheral nerve endings. Hence, the NE content of a particular tissue is a reflection of its sympathetic innervation. As a corollary to this, the absolute level of cathecholamine for a specific tissue remains contsant and is generally not subject to change under normal circumstances. However,

there is variation between tissue types. This relation holds true, despite variations in the level of sympathetic activity. Since NE is released from granules in response to nerve stimuli (or following the administration of indirect-acting sympathomimetic agents) a dynamic steady state must exist where catecholamine biosynthesis and utilization are in balance. Therefore, sympathetic activity should be assessed, not by measurement of steady state tissue levels of NE, but by measuring NE turnover using the techniques of steady state kinetics.

Of the various modalities available to measure turnover, one of the more popular, and the one used in this thesis, involves the use of tracer amounts of tritiated NE (^{3}H-NE). A tracer dose of ^{3}H-NE, intravenously administered, is taken up by the axonal membrane [60,61] and equilibrated with endogenous NE stores [62). It thus serves as a valid marker of NE release [63]. As noted before, alterations in adrenergic nerve activity effect concurrent changes in NE release and biosynthesis. Thus, following the introduction of tracer ^{3}H-NE, the intracellular concentration of label (or its specific activity) will decline as a function of time and adrenergic activity [64]. This rate of decline may be used to calculate the turnover rate of NE, and concomitantly, gauge the level of sympathetic activity.

Several authors have provided substantial theoretical and experimental support for this approach, and have utilized it in measuring levels of adrenergic activity in various organs under different conditions of stress. For example, Landsberg et al. demonstrated in Sprague-Dawley rats that increased "de novo" biosynthesis of catecholamines occurs coincidentally with augmented turnover [65]. Another study revealed increased NE turnover in the heart of male albino rats injected with ^{3}H-NE three hours before such stressful stimuli as cold, electric shock and immobilization [66].

Finally, Taubin et al. determined fractional turnover, half-life and turnover rate of ^{3}H-NE at various levels in the rat gastrointestinal tract [67]. They found the highest level of NE turnover to be in the duodenum and the colon. These areas also demonstrated the largest concentration of NE, as established by histochemical fluorescent studies.

The validity of these turnover experiments derives from a few very fundamental kinetic principals. In the paragraph to follow, a mathematical foundation for the kinetic analysis used in this thesis is presented.

First, it should be recalled that the tracer turnover technique takes advantage of the steady state kinetic principle that rates of synthesis and loss of catecholamines are equal. Moreover, the removal of NE is proportional to its concentration. Let K = rate of decline (or synthesis) of NE, $[NE]_t$= normal tissue amine concentration at time = t, and k = rate constant of NE efflux. Then $K = k[NE]_t$. Under steady state conditions, the decline in catecholamine concentration is expressed by the following relation: $-d\frac{[NE]}{dt} = k[NE]_t$ (1). Integrating (1) from time = 0 to time = t, one obtains: $\int_0^t \frac{d[NE]}{dt} = [NE]_t = [NE]_o e^{-kt}$ (2). Converting to $\log_{10}$, (2) becomes: $\log [NE]_t = \log [NE]_o - 0.434\ kt$, where $[NE]_o$ = initial level; $[NE]_t$ = level of amine at time = t. A plot of $\log [NE]_t$ vs. time yields a straight line with slope equal to -0.434 k, or the rate constant of NE efflux. Therefore $k[NE]_t$ = rate of NE efflux = rate of NE synthesis in the steady state. If $K = k[NE]_t$, then k is the fraction of total NE that is formed or lost per unit time, or fractional turnover. The reciprocal of k or k^{-1} or $[NE]_t/K$ is the time required for the biosynthesis of an amount of NE equal to that initially stored in the tissue, or the turnover time [68].

OBJECTIVES OF THE THESIS

As emphasized previously, a more realistic measure of sympathetic activity is reflected in the turnover rate of catecholamines, rather than by determinations of absolute tissue levels. Application of this principle, therefore, permits a more direct correlation of adrenergic activity and the various sympathetically mediated physiologic phenomena. Of particular interest to me, is the role played by the sympathetic nervous system in the metabolism of sodium by the kidney. Hence, my primary objective in pursuing this research was to demonstrate that renal catecholamine turnover did exist, and could be measured. It is hoped that further research will apply the methods developed and the results obtained here in such physiologic studies as the impact of sodium deprivation on renal catecholamine turnover. All such efforts should advance our understanding of the mechanisms behind non-aldosterone mediated tubular reabsorption of sodium.

MATERIALS AND METHODS

The quantity of intravenously administered ^{3}H-NE taken up by a tissue under normal conditions is a function of three major parameters: (1) the amount of sympathetic innervation characteristic for such a tissue [69], (2) the percentage of cardiac output delivered to the tissue, and (3) the level of adrenergic activity, or sympathetic tone, extant at the time of injection. Depending upon the organ under investigation, however, there may be modifications of, and additions to, the above considerations. For example, uptake by the brain is complicated by the presence of a blood brain barrier. The kidney also presents special difficulties in measuring catecholamine uptake and subsequent turnover. Following iv. injection of ^{3}H-NE, unincorporated ^{3}H-NE and its labeled catabolites collect in the

urine. These are a source of potential contamination when the kidneys are prepared for measuring ^{3}H-NE. Attempts were made to overcome this problem in the experiments to follow.

Female Sprague-Dawley rats (140-215 grams), were purchased from the Charles River Company, Wilmingham, Massachusetts. ^{3}H-L-NE was obtained from the New England Nuclear Corporation and purified prior to use by adsorption onto an alumina column at pH = 8.6. This was eluted from the resin with 0.2N acetic acid and preserved in a refrigerator with small amounts of ascorbic acid.

Tracer ^{3}H-NE was introduced into the rats by tail vein injection. At appropriate times, animals were sacrificed by a blow to the base of the skull and hearts and kidneys extirpated. These were prepared by either immediate homogenization in iced 0.4N $HClO_4$ (to preserve and release all the catecholamines), or by differential centrifugation in 0.25M iced sucrose (to be described) to obtain a vesicular fraction. The latter was then homogenized in iced 0.4N $HClO_4$. After acidification with perchlorate, precipitated protein was removed by centrifugation. NE was subsequently obtained from the supernatant by adsorption on alumina, previously equilibrated with sodium acetate pH 8.6 [70,71]. Radioactivity of ^{3}H-NE was determined by counting aliquots of alumina eluate for ^{3}H-NE by liquid scintillation spectometry on a Packard Tri-Carb scintillation counter (18 per cent efficiency). Attempts to determine endogenous NE were made by the trihydroxyindole method of Von Euler and Lishajake [72], (Aminco Bowman Spectrophoto-fluorometer). In turnover experiments, activity of the sympathetic nervous system with respect to time was measured in terms of muc ^{3}H-NE/gram tissue, and corrected for varying recovery from

the alumina column.*

Perfusion of kidneys in situ:

In an effort to remove all unincorporated catecholamine and labeled catabolites from tubular lumens prior to extirpation of the organ for analysis, an attempt was made to perfuse the kidneys with saline. The aorta was cannulated with a 21-gauge Butterfly needle, and ligatures placed above the take-off of renal arteries from the aorta and below the site of cannulation. Iced saline was then infused with a syringe. Samples of urine were taken from the bladder and used for scintillation counting, endogenous NE determinations, and measurement of creatinine.

Preparation of vesicles from sympathetic nerve endings in the kidney (Fig. 1):

Preparation of this subcellular fraction by differential centrifugation has been achieved in several laboratories [52,73]. In the experiments to follow, the extirpated organs were immediately chilled on crushed ice and then homogenized in 0.25M sucrose, 4°C. (At all times, close attention was paid toward keeping the preparation as near as 4°C as possible.) The homogenate was then centrifuged 10,000 X g for 15 minutes in a Sorvall R-11. Supernatant, Fr 1, containing vesicles, was subjected to centrifugation in a Spinco Model L ultracentrifuge at speeds of 100,000 X g for 1 hour, 4°C. Both pellet and supernatant obtained after this step were saved and treated

*As will be described later, attempts to measure enodgenous NE in the kidney were unsuccessful. Hence I was obliged to quantify adrenergic activity in terms of concentration of ^{3}H-NE, that is, muc ^{3}H-NE/gram tissue. The validity of this is based upon two assumptions. (1) Endogenous NE content varies among tissues within the animal. (2) Relative to each tissue, NE content/gram is approximately the same among animals of the species. Clearly, it would be more desirable to have measured endogenous NE so that specific activity, instead of concentration, could be used.

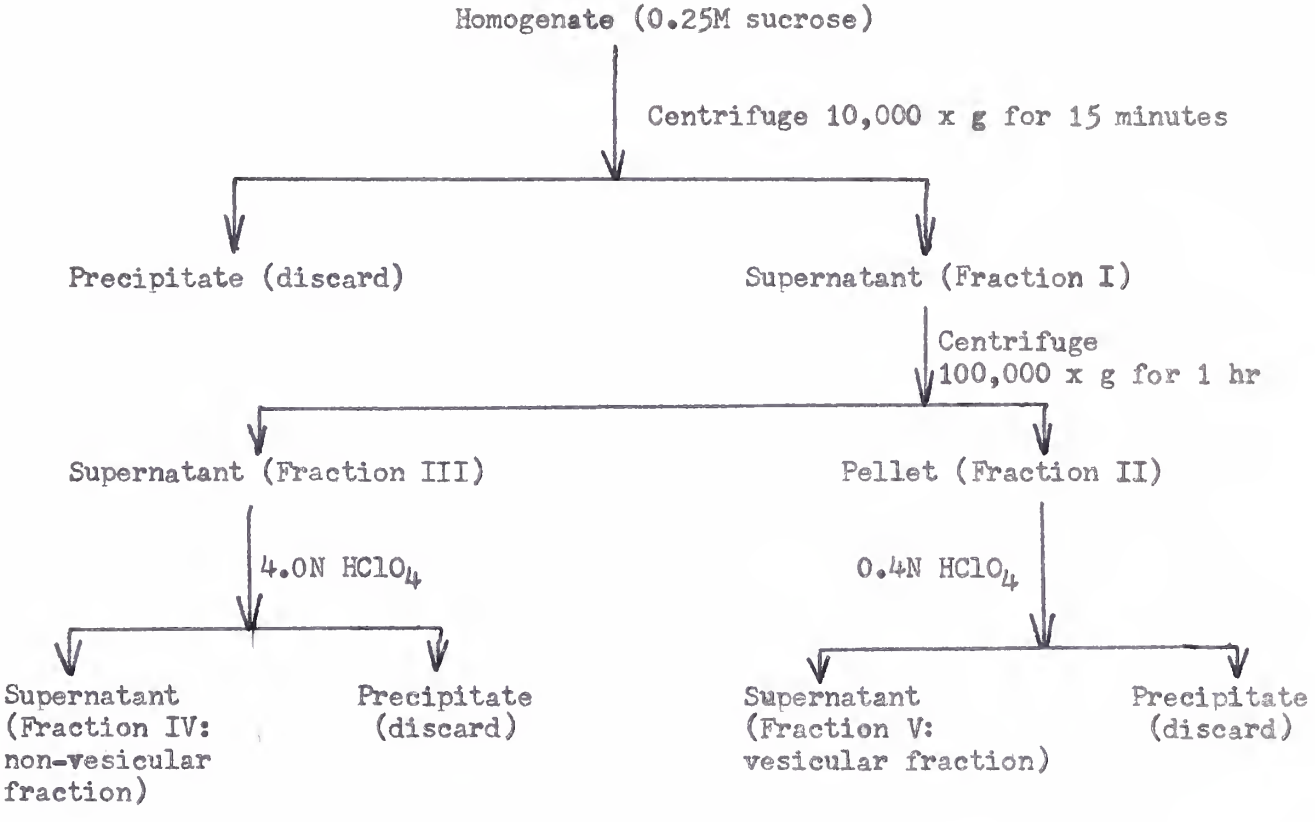


Fig. 1. Preparation of sympathetic neuronal vesicles by differential centrifugation

as follows.

NE in the supernatant (Fr III) was preserved by acidifying the preparation with 4.0 N $HClO_4$. Final volume was 13.0cc and concentration/ equal to 0.4N $HClO_4$. Proteinaceous precipitate was removed by centrifugation at low speed. The pellet (Fr II), containing vesicles (granules) and other microsomal components, was washed three times with 0.25M sucrose (4°C) and then resuspended in 0.4N $HClO_4$ (to free ^{3}H-NE). This preparation was re-homogenized, and centrifuged at low speed to obtain the supernatant, Fraction V (final volume = 10.0cc). Fractions IV and V were ultimately applied to an alumina column to allow separation of ^{3}H-NE from its labeled metabolites.

Vesicular preparation from rat heart:

The procedure was the same as that for the kidney, with the exception that interest was directed primarily toward the pellet (Fraction V).

Determination of nonspecific uptake or adsorption of ^{3}H-NE to granules from heart and kidney:

Hearts and kidneys from animals not administered a tracer dose of ^{3}H-NE were homogenized in 0.25M sucrose to which 50 µl of 280 µc/cc DL-^{3}H-NE had first been added. Supernatant (Fr IV) and granular (Fr V) preparations were obtained as outlined above and concentration of ^{3}H-NE determined after purification with alumina. Per cent nonspecific uptake was obtained using the following formula:

$$\text{Per cent} = \frac{[^3\text{H-NE}]\ \text{Fr V}}{[^3\text{H-NE}]_V + [^3\text{H-NE}]_{IV}}$$

Blockade of nonspecific uptake or adsorption of ^{3}H-NE:

In a series of experiments, heart and kidneys were homogenized at 4°C in 0.25M sucrose with ^{3}H-NE (0.65-0.87 µc/cc) and various agents known to inhibit uptake of NE by vesicles. Granular preparations were then obtained by differential centrifugation and ^{3}H-NE measured. Though several rats were

used, each served as its own control control since one kidney and one-half a heart were incubated with ^{3}H-DL-NE in the absence of inhibitors; while the other kidney and one-half heart were subjected to inhibition. Based on the per cent nonspecific uptake by the control, a per cent inhibition of uptake by the inhibitor could be ascertained. The agents and their concentrations were as follows:

(1) Reserpine (10^{-4}M) + EDTA (3×10^{-3}M)

(2) Nonisotopic DL-NE (1.47×10^{-6}M) + EDTA (3×10^{-3}M)

(3) Nonisotopic DL-NE (2.9×10^{-5}M), (1.2×10^{-4}M), (1.2×10^{-3}M)

Per cent uptake was calculated from the ratio, $\frac{[^{3}\text{H-NE}]_{V}}{[^{3}\text{H-NE}]_{V} + [^{3}\text{H-NE}]_{IV}}$, where V and IV refer to ^{3}H-NE in pellet and supernatant, respectively.

Turnover of ^{3}H-NE in rat heart and kidneys:

Twenty Sprague-Dawley female rats (137-169 grams) were divided into groups of five, and ^{3}H-L-NE/(400 μc/kg) administered by tail vein injection. At intervals of one-half, 3, 7, and 12 hours respective groups were sacrificed by a blow to the base of the skull. Organs were rapidly removed, blotted dry, and weighed. (Extreme care was taken to ensure that kidneys were free of adrenal gland tissue.) Each animal was treated in the following manner: (1) One kidney was immediately homogenized in 0.4N $HClO_4$ (4°C) ("perchlorate kidney"), while a granular preparation was obtained from the other. (2) The hearts were divided in half, with one portion set aside for immediate homogenization in 0.4N $HClO_4$ ("perchlorate heart"); and the other used to prepare vesicles. Aliquots were obtained prior to, and following passage over the alumina column for counting via scintillation spectrometry.

Effect of ganglionic blockage on catecholamine turnover:

Twelve female Sprague-Dawley rats were injected intravenously with

^{3}H-L-NE at a dose of 420 μg/kg. At 5 minutes and 3 hours after administration of ^{3}H-NE, half the animals were given chlorisondamine hydrochloride (15 mg/kg) intraperitoneally. The remaining six served as controls and were injected with saline, ip, at the above times. Six hours post ^{3}H-NE injection, the animals were sacrificed and kidneys and hearts were removed. A vesicular preparation (Fr V, IV) was obtained from the kidneys. However, the hearts were merely blotted dry and homogenized in 0.4N $HClO_4$. Concentration of ^{3}H-NE (mμc/g) was determined before and after application to alumina.

Kinetic analysis:

In the introduction it was pointed out that under steady state conditions, the removal and formation of catecholamine are in balance. Moreover, the disappearance of catecholamine is proportional to its concentration at any time. That is, $-d[NE]_t/dt = k[NE]_t$, where k is the rate constant of NE efflux. This relationship is an exponential one, describes the curve $Y = ab^x$, and involves a constant ratio of change--a constant ratio of change in the amount of change. If b is greater than 1, the trend is upward; and the amount of change is undergoing a constant percentage increase. Conversely, it b is less than 1, there is described a downward trend, with the amount of change showing a constant percentage of decrease. No change results if b = 1.

This exponential relation may be made linear by converting to logarithms: $\log Y = \log a + x \log b$. Here the slope is represented by log b and y-intercept, by log a. Thus, to calculate the percentage increase or decrease of an exponential function, one need merely determine the slope, log b; find the antilog b; and then subtract this from 1 (or subtract 1 from this).

In the turnover experiment, log y is concentration ^{3}H-NE (mμc/g) and x represents time. By the method of least squares, log y was plotted vs time and a line of regression obtained. This was plotted on semilog paper with the standard error about the mean concentration of ^{3}H-NE for each time interval. The slope (log b) is easily determined and used to calculate the rate constant of decline (per cent of pool declining per hour), k, or fractional turnover. Turnover time is obtained by finding the reciprocal of k and multiplying by 100. Finally, half-life of NE is calculated by dividing 0.693 by k, and then multiplying by 100.

Statistical analyses were used to determine the coefficient of correlation between decline in concentration of ^{3}H-NE and time for each preparation. Moreover, the standard t-test was utilized to establish the effect of chlorisondamine on turnover of catecholamine.

RESULTS

1. Measurement of endogenous NE in kidneys and heart, using whole organ homogenates

Unfortunately, the fluorimetric assay of renal endogenous NE (after passage of homogenates over alumina) was repeatedly frustrated by high blank readings. Apparently a nonproteinaceous material resides within the kidney, is eluted from alumina along with NE, and reacts in the trihydroxyindole procedure to obscure the fluorescence of catecholamine. It is of note that this substance is absent in the urine. See Figure 2 and Tables 1 and 2.

An attempt to side-step this difficulty was made by applying the alumina column eluate to a Dowex cation-exchange resin. Blank readings were much diminished; however, there was little difference between blanks and sample readings, making calculations somewhat unreliable. Table 3.

This is most likely attributable to the large dilution factor encountered using two exchange columns.

Vigorous effort was devoted toward refining this two-column procedure; but fluorescence readings continued to be so low that consistent determinations could not be made. Some of our results indicated endogenous renal NE concentration of from 50-707 ng/g tissue. Hence, it was felt that although it appeared possible to make determination of endogenous NE by the two column method, the results were not reliable enough to justify use of this method in the experiments to follow. Instead, the decision was made to measure sympathetic activity in terms of mμc ^{3}H-NE/gram tissue, rather than per ng NE--a less meaningful quantity, but not so much so as to jeopardize the validity of our results.

II. Perfusion of rat kidneys in situ

Several attempts were made to perfuse the kidneys in situ with saline in order to remove unincorporated ^{3}H-NE (and labeled catabolites from tubular lumens. Clearly, it is not desirable to have these "contaminants" contributing to the determination of ^{3}H-NE taken up by the sympathetic nerve endings.

The perfusion attempts, however, were exceedingly time consuming and did not effect a free flow of urine. Rather than persist in this endeavor, the decision was made at this juncture to attempt purification of the vesicles. For it is in the vesicles that incorporated ^{3}H-NE would be stored, prior to subsequent release. There is, therefore, theoretical justification for pursuing this line of attack.

III. Determination of endogenous NE of vesicles and their nonspecific uptake of ^{3}H-NE

The properties of sympathetic nerve vesicles from the kidney have

Fig. 2

Formula for calculation of endogenous norepinephrine:

a. RI_s (fluorescent reading for internal standard) = amount of fluorescence for 100 ng norepinephrine added + amount of fluorescence due to NE in 0.5cc eluate (RI_s = fluorescence of 100ng NE + x)

b. x = fluorescence of x' ng NE in eluate

c. b = fluorescence of blank

d. CF = correction factor for % recovery of NE from column, aliquots removed for various measurements etc.

$$\frac{x' \text{ ng NE}}{100 \text{ ng NE}} = \frac{x - b}{(RI_s - b) - (x - b)}$$

$$x' \text{ ng NE} = (100 \text{ ng NE}) \frac{(x - b)}{(RI_s - x)} \quad (CF)$$

Rat #	Weight of Rat(g)	Combined wt. of kidneys g	Vol. urine from bladder cc *	Dose ^{3}H-NE ug/kg iv.
1	260	2.31	-	233
2	262	2.80	0.2	233
3	231	2.54	0.2	100
4	231	1.67	0.2	100
5	200	1.86	0.2	100

* 0.2 cc urine diluted to 5.0cc with 0.4N $HClO_4$

Table 1. Determination of endogenous NE in alumina eluate from rat kidneys and urine, in a typical experiment — experimental set-up for values obtained in Table 2.

Sample	RI_s	x	x	b	Endogenous NE (ng)	Conc ^{3}H-NE (muc/g)
Rat# 1	94	40	37	120	-	99.5
# 2	108	51	54	100	-	89.0
# 3	92	38	39	116	-	33.2
# 4	90	41	36	123	-	51.4
# 5	86	40	39	102	-	93.6
Std 1*	138	55	53	10	552	-
2	145	67	64	8	760	-
3	140	65	65	6	819	-
U 2+	73	10	10	7	66	-
3	84	9	8	9	-	-
4	86	22	17	5	364	-
5	90	14	8	10	21	-

* Std 1,2,3 : 1000 ng DL NE applied to three alumina columns so that % recovery of NE off columns could be calculated.

+ U 2,3,4,5 : refers to values obtained from urine aliquots of rats 2,3,4,5

Table 2. Values obtained in fluorescence assay for endogenous NE from kidneys and urine of rats described in Table 1. Renal concentration of ^{3}H-NE determined by scintillation spectrometry also presented.

Sample	RI_s	x	x	b	ng NE	ng NE/g tissue
Rat # 1	95	14	12	6	1633	707
2	99	12	12	14	-	-
3	88	3	5	15	-	-
4	90	13	10	13	-	-
5	100	11	10	8	438	236
Std # 1	100	13	10	14	-	-
2	100	14	15	11	113	-
3	105	12	17	7	230	-

Table 3. Fluorescence assay for endogenous NE after passage of alumina column eluate (Table 2) over a Dowex cation exchange resin.

not been described. One such property of some importance in future turnover experiments is the extent to which a granular preparation nonspecifically adsorbs ^{3}H-NE. An approach to this problem is outlined in Methods, and the results presented in Table 4.

From these data it became clear that nonspecific uptake, or adsorption, of ^{3}H-NE by the vesicles did occur. These findings were not surprising, as they are in accordance with those of Potter and Gillis.

An effort was also made to measure endogenous NE in vesicular preparations after elution from alumina. Both kidneys from a rat were pooled and granules prepared according to Methods. Results of the fluorimetric assay, Table 5, demonstrate NE levels so low (or blanks so high) as to preclude reliable calculations of endogenous NE.

IV. Blockage of nonspecific uptake or adsorption of ^{3}H-NE by the vesicles

Several agents have been employed to block this nonspecific uptake (or adsorption), some more successfully than others--depending upon the tissue. Among the inhibitors used are reserpine, EDTA, and competetive inhibition in unlabeled NE. As outlined in Methods, vesicular preparations were incubated with these agents.

A. Effect of 10^{-4}M reserpine + 3×10^{-3}M EDTA (Tables 6 and 7):

Virtually no heart pellet could be retrieved from the preparation incubated with reserpine and EDTA. Similarly, pellets from kidneys incubated with reserpine and EDTA were smaller than controls. Were it not for this observation, one might have concluded that 10^{-4}M reserpine + 3×10^{-3}M EDTA inhibited nonspecific uptake in the kidney by 99.14 per cent. The control study revealed 5.66 per cent and 1.31 per cent uptake by kidney and heart, respectively, at 4°C.

Tissue	Rat#	Wt. of tissue (g)	Concentration ^{3}H-NE - Fr V (muc/g)*	Conc. ^{3}H-NE Fr IV (muc/g)**	% non-specific uptake V/(V + IV)
Kidney	1	1.7536	318	10043	3.1
	2	1.5020	391	9964	3.8
	3	1.3708	385	10199	3.7
Heart	4	0.7619	68	432	13.0

*Granular Fraction V after passage over alumina
**Supernatant Fraction IV after passage over alumina

Table 4. Non-specific uptake, or adsorption, of ^{3}H-NE by kidney and heart vesicles

Tissue	Rat#	Wt of tissue (g)	RI_s	x	x	b
Kidney	1	1.7536	96	10	9	10
	2	1.9580	87	9	8	11
	3	2.0002	93	8	7	8
	4	1.5850	86	10	10	11
	5	1.6100	93	7	8	8
	6	1.8085	92	12	7	8
	7	1.7860	85	10	10	9
	8	1.7230	93	9	8	8
	9	1.5020	86	9	16	8
	10	1.3708	90	8	8	9
Heart	2	0.7619	93	8	5	5
Standard*	1		140	60	60	4
	2		150	64	65	6
	3		130	64	63	3

*Standard 1,2,3: 1000 ng DL-NE applied to three columns so that % recovery of NE off columns could be calculated

Table 5. Fluorometric assay of endogenous NE content of neuronal vesicles from rat kidney and heart

Tissue	Rat#	Wt. tissue (g)	^{3}H-NE Fr V (muc/g)	^{3}H-NE Fr IV (muc/g)	% uptake V/(V + IV)	Avg. % uptake
Kidney	1	0.78	594	7583	0.073	5.66
	2	0.79	344	7289	0.045	
	3	0.77	514	7107	0.067	
	4	0.75	460	7573	0.057	
	5	0.72	418	7630	0.052	
	6	0.85	346	7279	0.045	
Heart	1	0.30	60	8861	0.007	1.31
	2	0.30	36	8833	0.004	
	3	0.26	120	8442	0.014	
	4	0.26	103	8226	0.012	
	5	0.23	268	8444	0.031	
	6	0.29	97	8824	0.011	

Table 6. Control. Uptake of ^{3}H-NE in the absence of reserpine and EDTA

Tissue	Rat#	Wt. tissue (g)	^{3}H-NE Fr V (muc/g)	^{3}H-NE Fr IV (muc/g)	%uptake V/(V + IV)	Avg. % uptake
Kidney	1	0.74	47	7718	0.006	0.86
	2	0.80	47	7683	0.006	
	3	0.82	67	7572	0.009	
	4	0.79	58	7383	0.008	
	5	0.77	75	7760	0.010	
	6	0.85	102	7652	0.013	
Heart	1	0.25	9	6330	0.000	0.00
	2	0.34	11	9173	0.000	
	3	0.27	15	9743	0.000	
	4	0.24	4	8995	0.000	
	5	0.30	26	9274	0.000	
	6	0.29	11	9438	0.000	

Table 7. Experimental. Uptake of ^{3}H-NE in the presence of 10^{-4}M reserpine and 3 X 10^{-3}M EDTA

From this experiment, it was concluded that either reserpine or EDTA or both destroyed the vesicles or dramatically altered their sedimentation properties.

B. Effect of competitive inhibition with unlabeled NE (1.46×10^{-6}M) + 3×10^{-3}M EDTA (Tables 8 and 9):

In view of the above findings, a similar experiment was performed using EDTA as a noncompetitive inhibitor and unlabeled NE as a competitive inhibitor. Results are portrayed in Tables 8 and 9.

Again, no heart pellet was obtained from the preparation incubated with EDTA. Clearly, this chelating agent plays a role in either the destruction of vesicles or alteration of their sedimentation characteristics. As in the above experiment, the average uptake at 4°C of ^{3}H-NE by kidney and heart controls was 5.75 per cent and 0.92 per cent respectively.

C. Effect of competitive inhibition with unlabeled NE alone--2.8×10^{-5}M, 1.1×10^{-4}M, 1.2×10^{-3}M (Tables 10 and 11):

The data show no significant competitive inhibition by NE of vesicular nonspecific uptake of ^{3}H-NE.

These experiments indicated that kidney and heart granules are capable of either passively or actively adsorbing small amounts of ^{3}H-NE. Furthermore, efforts to block this phenomenon were unsuccessful. With this in mind, the decision was made to proceed with turnover experiments. However, the results obtained would have to be interpreted in light of this finding.

V. Turnover of catecholamine in rat heart and kidney

Several preliminary experiments indicated that the concentration of ^{3}H-NE taken up by renal sympathetic neurons following iv. administration declined with time. These impressions led to the formal turnover experiment described in Methods.

Tissue	Rat#	Wt. tissue (g)	^{3}H-NE Fr V (muc/g)	^{3}H-NE Fr IV (muc/g)	% uptake V/(V + IV)	Avg. % uptake
Kidney	1	0.71	436	6880	0.059	5.75
	2	0.69	463	7000	0.062	
	3	0.70	373	6980	0.050	
Heart	1	0.27	91	21800	0.004	0.92
	2	0.32	166	17900	0.009	
	3	0.32	270	18200	0.014	

Table 8. Control. Uptake of ^{3}H-NE in the absence of EDTA (3×10^{-3}M) and unlabeled NE (1.46×10^{-6}M)

Tissue	Rat#	Wt. tissue	^{3}H-NE Fr V (muc/g)	^{3}H-NE Fr IV (muc/g)	% uptake V/(V + IV)	Avg. % uptake
Kidney	1	0.78	81	6520	0.012	1.28
	2	0.73	90	7290	0.012	
	3	0.68	118	2830	0.014	
Heart	1	0.29	0	20750	0.000	0.00
	2	0.23	0	27500	0.000	
	3	0.33	0	17550	0.000	

Table 9. Experimental. Uptake of ^{3}H-NE by vesicles in the presence of 3×10^{-3}M EDTA and unlabeled NE (1.46×10^{-6}M)

Tissue	Rat#	Wt. tissue (g)	Conc. DL-NE	^{3}H-NE Fr V (muc/g)	^{3}H-NE Fr IV (muc/g)	uptake % V/ (V + IV)	Avg% uptake
	1	0.74	0.00M	423	5950	0.006	
	2	0.77		401	5920	0.063	
	3	0.82		320	5440	0.055	
	4	0.82		213	4960	0.041	
Kidney	5	0.90		176	5360	0.032	4.51
	6	0.95		173	4365	0.038	
	7	0.77		204	6620	0.030	
	8	0.77		255	5450	0.044	
	9	0.74		222	5960	0.036	
	1	0.28	0.00M	154	18050	0.008	
	2	0.27		72	18300	0.004	
	3	0.30		43	17950	0.002	
	4	0.34		51	16930	0.003	
Heart	5	0.30		132	15900	0.008	0.60
	6	0.34		112	14500	0.008	
	7	0.35		109	17050	0.006	
	8	0.37		119	13550	0.009	
	9	0.28		137	22090	0.006	

Table10. Control. Non-specific uptake of ^{3}H-NE by vesicles in absence of competitive inhibition by unlabeled NE

Tissue	Rat#	Wt. tissue (g)	Conc. DL-NE	^{3}H-NE Fr V (muc/g)	^{3}H-NE Fr IV (muc/g)	uptake % V/(V + IV)	Avg % uptake
	1	0.80	2.8×10^{-5}	340	5590	0.056	
	2	0.70		428	6830	0.059	5.70
	3	0.87		313	5150	0.057	
	4	0.79	1.1×10^{-4}	217	5350	0.039	
Kidney	5	0.82		263	6080	0.041	4.10
	6	0.98		198	4385	0.043	
	7	0.76	1.2×10^{-3}	151	5920	0.025	
	8	0.82		159	5240	0.029	2.60
	9	0.67		165	6520	0.025	
	1	0.30	2.8×10^{-5}	142	18000	0.007	
	2	0.26		82	19660	0.004	1.20
	3	0.27		16	19700	0.001	
	4	0.29	1.1×10^{-4}	76	17650	0.004	
Heart	5	0.26		128	19230	0.006	0.50
	6	0.28		119	18860	0.006	
	7	0.34	1.2×10^{-3}	73	17200	0.004	
	8	0.33		53	15200	0.003	0.30
	9	0.28		16	19130	0.001	

Table 11. Experimental. Competitive inhibition of non-specific vesicular uptake of ^{3}H-NE by unlabeled NE

A. Table 12 supplements the experimental design described in Methods, vis-a-vis weights of animals, organs, and volume of ^{3}H-NE injected.

B. Table 13 presents the raw turnover data from which kinetic parameters are derived.

C. From the information provided in Table 13, the mean ($\bar{x}$), standard deviation (SD), and standard error (SE), with respect to concentration of ^{3}H-NE, has been calculated as a function of time. Tables 14-18.

D. Calculation of the statistical significance of decline in concentration of ^{3}H-NE as a function of time. Table 19.

The statistical significance of decline in concentration of ^{3}H-NE with respect to time, for each preparation, was ascertained by determining a coefficient of correlation. The results indicate that except for the heart vesicles (Hv), there appears to be a significant r-value for each preparation. This is more clearly represented by constructing linear least square lines. Figures 3-7. In each figure two lines of regression are plotted: one determined from the concentration of ^{3}H-NE before passage over alumina; and one determined following passage over alumina. In addition, $\bar{x} \pm 2SE$ is included for each time point. The fractional turnover, turnover time, and half-life of ^{3}H-NE for each preparation are included in Table 20.

From the coefficients of correlation, lines of regression, and the kinetic parameters derived therefrom, a number of observations can be made.

(1) It is clear that the heart vesicular preparation did not adequately reflect turnover. In all likelihood, this is in part due to

Rat#	Wt. Rat (g)	K *	K_v **	H†	H_v‡	Vol. ^{3}H-NE⊙ (cc)
1	149	0.66	0.65	0.26	0.26	0.15
2	150	0.62	0.61	0.21	0.22	0.15
3	145	0.59	0.60	0.23	0.24	0.14
4	146	0.56	0.58	0.21	0.23	0.15
5	142	0.56	0.52	0.23	0.20	0.14
6	137	0.51	0.51	0.22	0.21	0.14
7	155	0.70	0.71	0.23	0.27	0.15
8	140	0.58	0.56	0.24	0.22	0.14
9	144	0.58	0.57	0.20	0.23	0.14
10	158	0.75	0.75	0.29	0.25	0.16
11	142	0.54	0.56	0.21	0.27	0.14
12	169	0.69	0.74	0.27	0.31	0.17
13	160	0.66	0.64	0.25	0.29	0.16
14	137	0.57	0.53	0.22	0.25	0.14
15	145	0.60	0.62	0.27	0.28	0.14
16	138	0.58	0.56	0.23	0.29	0.14
17	147	0.67	0.66	0.28	0.26	0.15
18	157	0.63	0.67	0.27	0.25	0.16
19	143	0.71	0.66	0.26	0.26	0.14
20	138	0.58	0.60	0.23	0.24	0.14

K* : Weight (g) of the kidney processed in 0.4N $HClO_4$ immediately following extirpation (referred to as "perchlorate kidney")

K_v** : Weight (g) of the kidney from which granules were obtained

H† : Weight (g) of the portion of heart processed in 0.4N $HClO_4$ immediately following extirpation (referred to as "perchlorate heart")

H_v‡ : Weight (g) of the portion of heart from which granules were obtained

⊙ : Dose of ^{3}H-L-NE was 400 uc/kg in a solution of 400 uc/cc.

Table 12.

Rat#	Time (hrs)	Before alumina					After alumina				
		Heart		Kidney			Heart		Kidney		
		H_v	H	K_v	K	K_s	H_v	H	K_v	K	K_s
20	½	1534	5198	80	864	608	1298	3468	70	255	128
19		1007	3530	63	615	438	742	2427	56	152	78
18		677	2768	31	415	312	696	2180	26	87	35
17		886	3854	86	768	489	804	2633	70	233	106
16		888	3993	51	548	428	761	2745	40	142	75
15	3	559	2462	24	142	82	441	1744	21	52	26
14		579	3167	50	283	132	741	1938	45	118	53
13		642	2672	29	164	103	876	1667	22	58	28
12		554	2598	24	153	76	470	1558	22	59	26
11		420	2067	32	174	105	628	1355	26	64	36
10	7	530	1547	30	98	53	424	1317	27	70	30
9		677	1972	23	100	56	470	1425	22	60	26
8		468	1479	20	84	52	446	1044	18	44	23
7		894	2040	25	117	62	1061	1464	24	81	29
6		877	2985	30	156	100	809	2419	32	78	39
5	12	559	1678	16	64	40	548	1082	13	48	20
4		924	2359	17	88	40	1014	1502	16	41	17
3		421	1827	9	59	28	491	1095	8	26	9
2		651	2194	23	109	54	768	1339	18	50	20
1		382	1414	10	51	29	400	930	11	27	10

H_v : refers to the concentration of ^{3}H-NE (muc/g) in the heart vesicular preparation (Fr V)

H : refers to the concentration of ^{3}H-NE (muc/g) in the heart perchlorate prep.

K_v : refers to ^{3}H-NE (muc/g) in the kidney vesicular preparation (Fr V)

K : refers to ^{3}H-NE (muc/g) in the kidney perchlorate preparation

K_s : refers to ^{3}H-NE (muc/g) in the kidney supernatent prep. (Fr IV)

Table 13.

	Time (hrs)	$\bar{x}$	SD	SE	$\bar{x}$ + 2SE	$\bar{x}$ - 2SE
Before Alumina	0.5	998	322	144	1286	710
	3.0	550	81	36	622	478
	7.0	689	194	87	863	515
	12.0	587	216	97	781	393
After Alumina	0.5	860	247	111	1082	638
	3.0	631	183	82	795	467
	7.0	642	282	126	894	390
	12.0	644	247	110	864	424

Table 14. Heart vesicles (values in terms of muc/g)

	Time (hrs)	$\bar{x}$	SD	SE	$\bar{x}$ + 2SE	$\bar{x}$ - 2SE
Before Alumina	0.5	3868	881	395	4658	3078
	3.0	2593	396	177	2947	2239
	7.0	2004	601	269	2542	1466
	12.0	1894	383	171	2236	1552
After Alumina	0.5	2690	484	217	3124	2256
	3.0	1652	216	97	1846	1458
	7.0	1533	521	233	1999	1067
	12.0	1189	227	102	1393	985

Table 15. Heart - perchlorate (values in terms of mue/g)

	Time (hrs)	$\bar{x}$	SD	SE	$\bar{x}$ + 2SE	$\bar{x}$ - 2SE
Before Alumina	0.5	62	22	10	82	42
	3.0	32	11	5	42	22
	7.0	25	4	2	29	22
	12.0	15	6	3	21	9
After Alumina	0.5	52	19	9	70	34
	3.0	27	10	4	35	19
	7.0	25	5	2	29	21
	12.0	13	4	2	17	9

Table 16. Kidney vesicles (values in terms of muc/g)

	Time (hrs)	$\bar{x}$	SD	SE	$\bar{x}$ + 2SE	$\bar{x}$ - 2SE
Before Alumina	0.5	642	177	79	800	484
	3.0	183	57	25	233	133
	7.0	111	27	12	135	87
	12.0	74	23	10	94	54
After Alumina	0.5	173	69	31	235	111
	3.0	70	27	12	94	46
	7.0	66	15	7	80	52
	12.0	38	11	5	48	28

Table 17. Kidney perchlorate (values in terms of muc/g)

	Time (hrs)	$\bar{x}$	SD	SE	$\bar{x}$ + 2SE	$\bar{x}$ - 2SE
Befire Alumina	0.5	455	107	48	551	359
	3.0	99	22	10	119	79
	7.0	64	20	9	82	46
	12.0	38	10	5	48	28
After Alumina	0.5	84	35	16	116	52
	3.0	33	12	5	43	23
	7.0	29	6	3	35	23
	12.0	15	5	3	21	9

Table 18. Kidney supernatent (values in terms of muc/g)

	Preparation	Coefficient of Correlation(r)
Before Alumina	Heart vesicles	-0.4194
	Heart perchlorate	-0.7362
	Kidney vesicles	-0.8319
	Kidney supernatant	-0.8777
	Kidney perchlorate	-0.8737
After Alumina	Heart vesicles	-0.2863
	Heart perchlorate	-0.7755
	Kidney vesicles	-0.8123
	Kidney supernatant	-0.7291
	Kidney perchlorate	-0.7898

Table 19.

	Preparation	Fractional Turnover *	Turnover Time (hrs)	Half-life ^{3}H-NE (hrs)
Before Alumina	Heart vesicles (H_v)	3.22	31.06	21.52
	Heart perchlorate (H)	5.68	17.60	12.20
	Kidney vesicles (K_v)	10.63	9.41	6.52
	Kidney perchlorate(K)	15.67	6.38	4.42
	Kidney supernatant(K_s)	17.49	5.72	3.96
After Alumina	Heart vesicles	2.16	46.30	32.08
	Heart perchlorate	6.09	16.42	11.38
	Kidney vesicles	9.95	10.05	6.96
	Kidney perchlorate	10.44	9.58	6.64
	Kidney supernatant	14.85	6.73	4.67

* % decline/hour

Table 20

Fig. 3

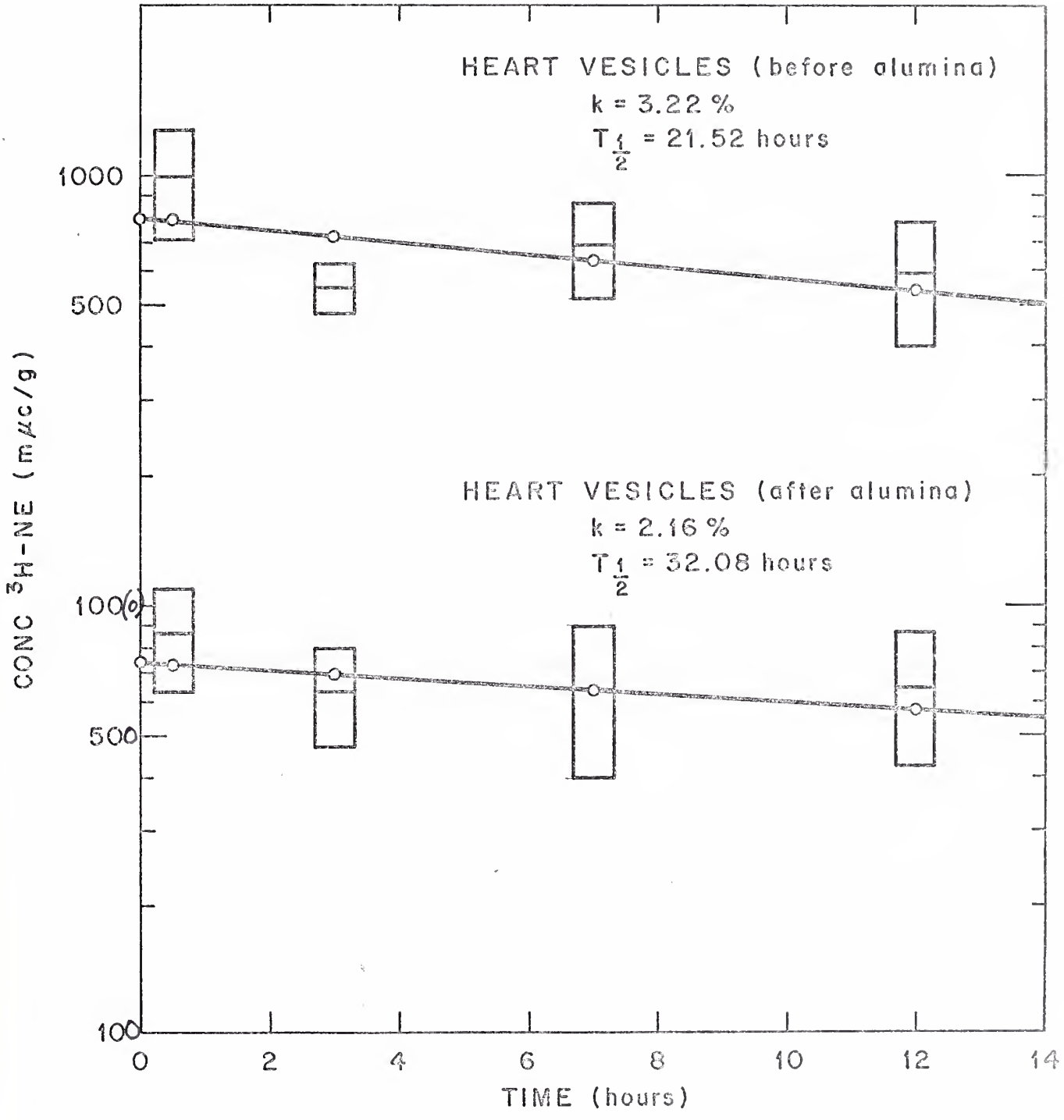

HEART VESICLES (before alumina)
k = 3.22 %
T 1/2 = 21.52 hours
HEART VESICLES (after alumina)
k = 2.16 %
T 1/2 = 32.08 hours
CONC 3H-NE (mμc/g)
1000
500
100(0)
500
100
0
2
4
6
8
10
12
14
TIME (hours)

Fig. 4

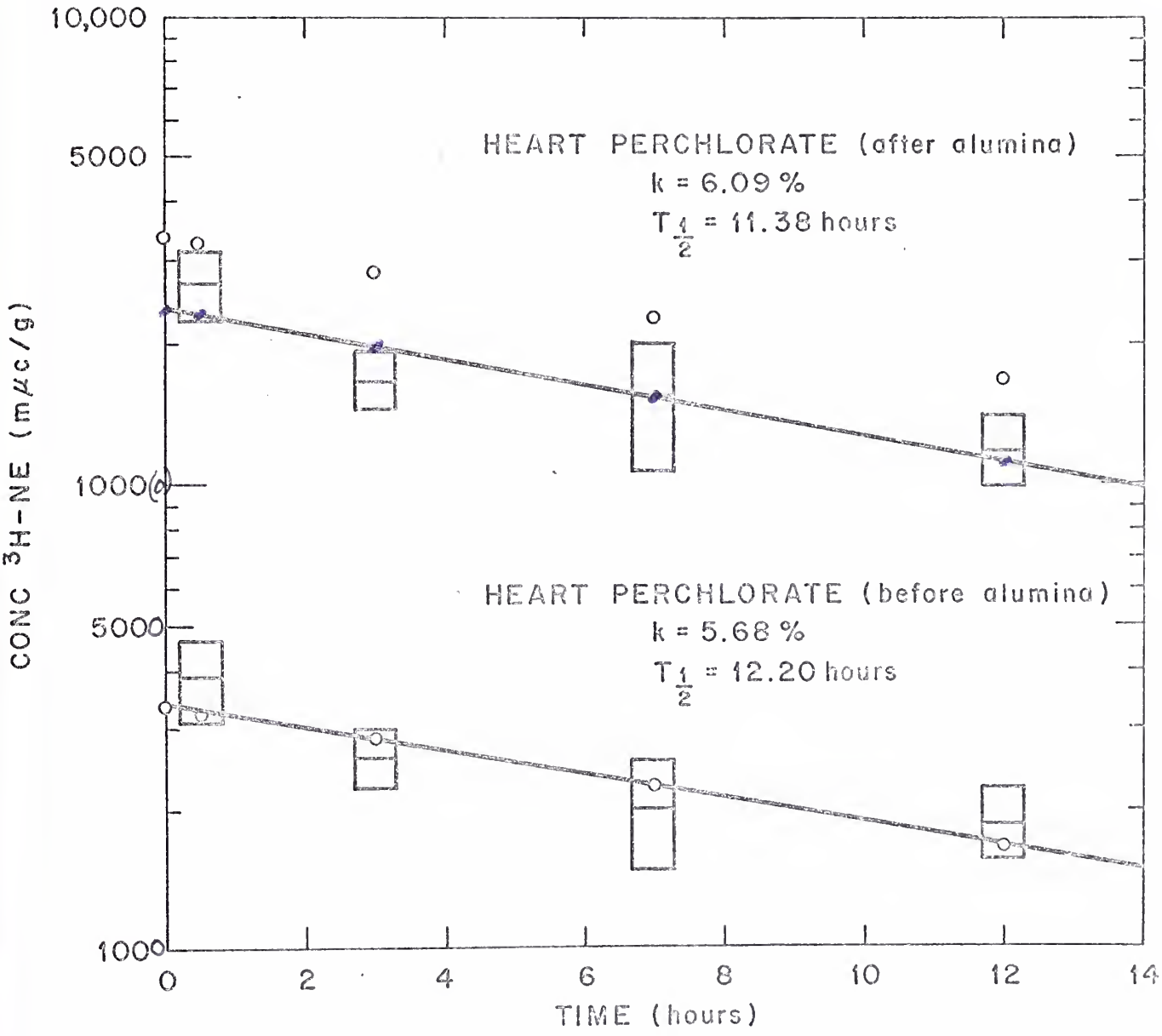

10,000
5000
1000
5000
1000
HEART PERCHLORATE (after alumina)
k = 6.09 %
T 1/2 = 11.38 hours
HEART PERCHLORATE (before alumina)
k = 5.68 %
T 1/2 = 12.20 hours
CONC 3H-NE (mμc/g)
0
2
4
6
8
10
12
14
TIME (hours)

Fig. 5

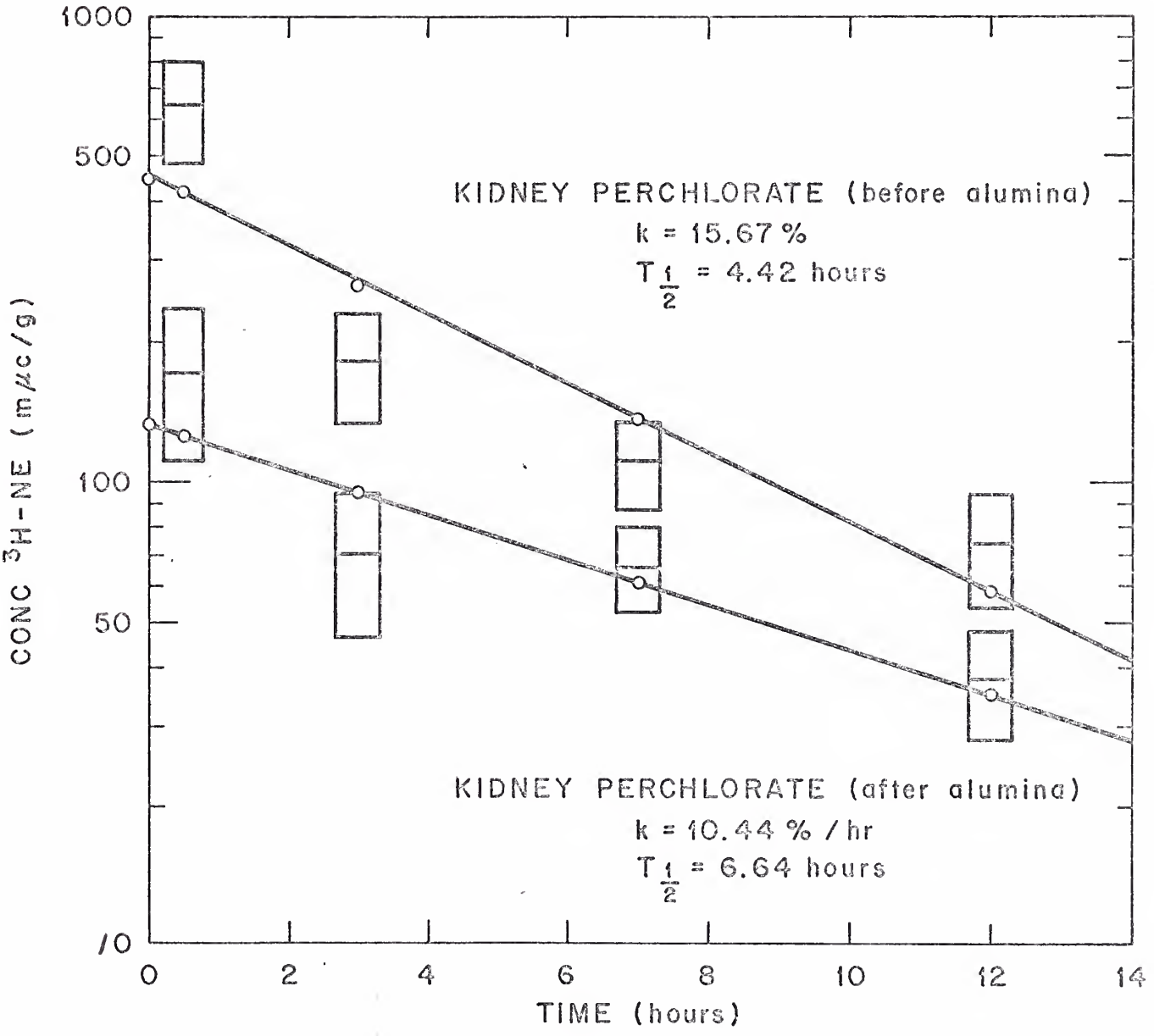

1000
500
100
50
10
CONC ^{3}H-NE (mμc/g)
KIDNEY PERCHLORATE (before alumina)
k = 15.67 %
$T_{\frac{1}{2}}$ = 4.42 hours
KIDNEY PERCHLORATE (after alumina)
k = 10.44 % / hr
$T_{\frac{1}{2}}$ = 6.64 hours
0
2
4
6
8
10
12
14
TIME (hours)

Fig. 6

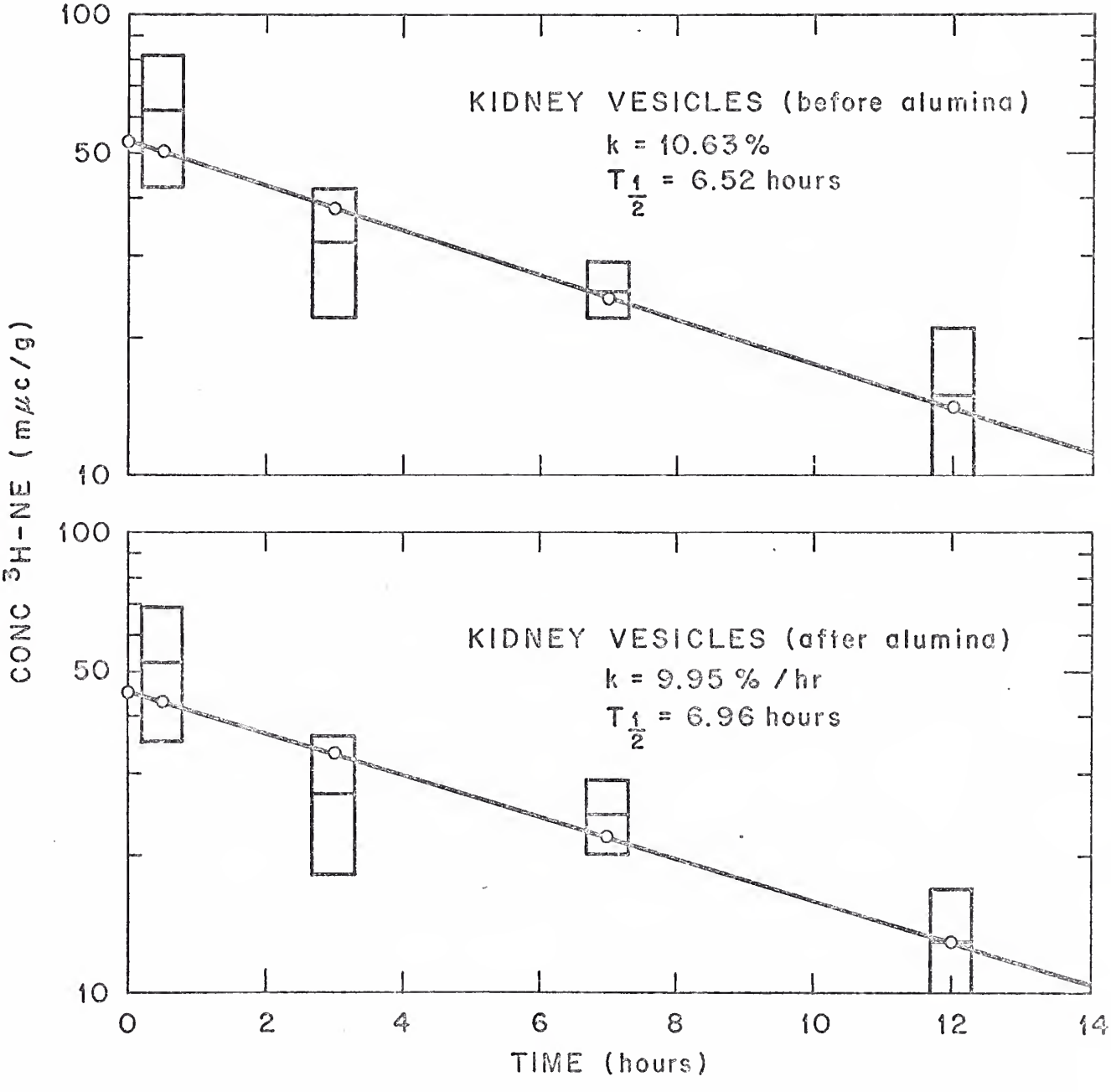
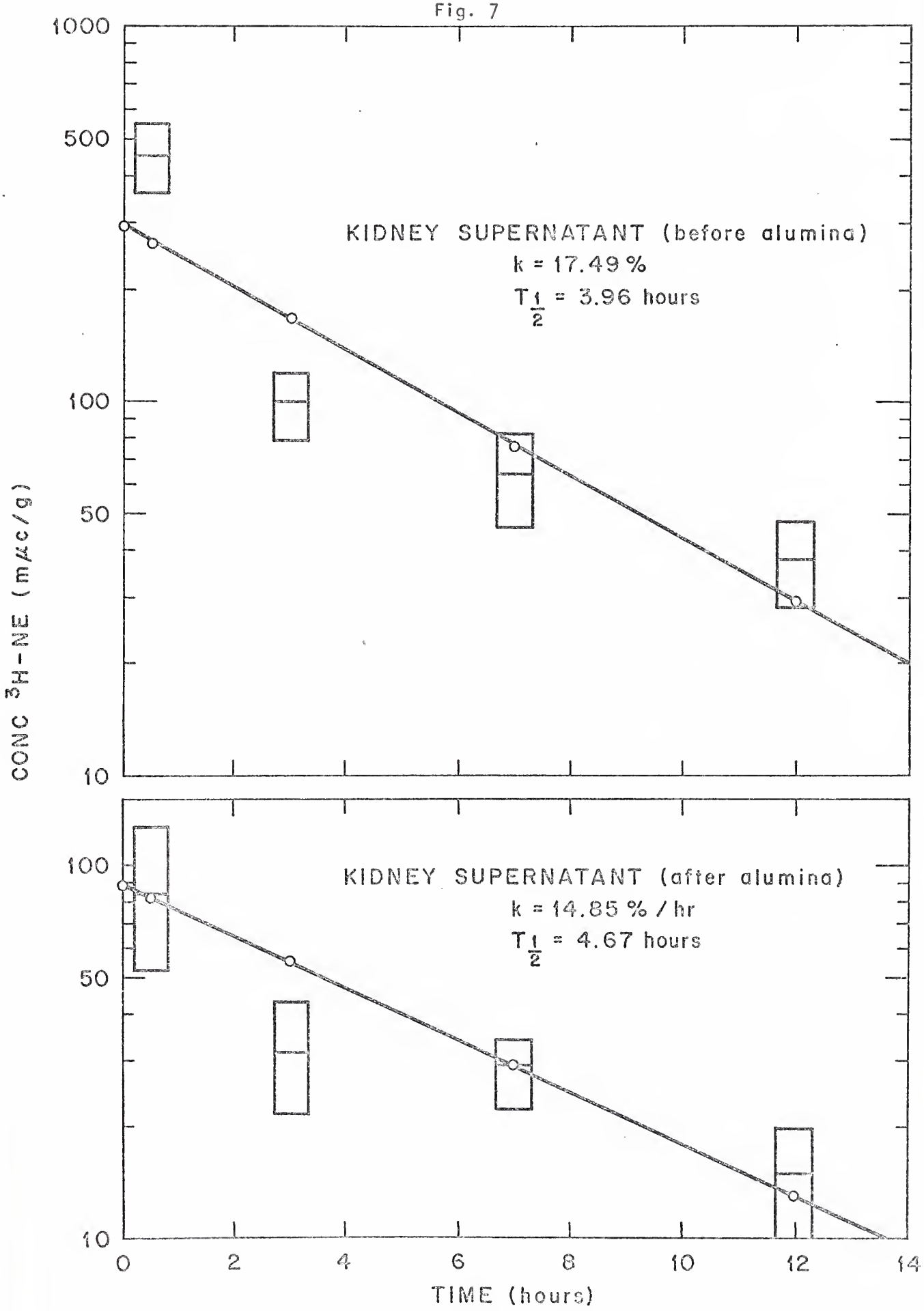

KIDNEY VESICLES (before alumina)
k = 10.63 %
T 1/2 = 6.52 hours
KIDNEY VESICLES (after alumina)
k = 9.95 % / hr
T 1/2 = 6.96 hours
CONC 3H-NE (mμc/g)
100
50
10
0
2
4
6
8
10
12
14
TIME (hours)

Fig. 7


1000
500
100
50
10
KIDNEY SUPERNATANT (before alumina)
k = 17.49 %
T 1/2 = 3.96 hours
CONC 3H-NE (mμc/g)
100
50
10
KIDNEY SUPERNATANT (after alumina)
k = 14.85 % / hr
T 1/2 = 4.67 hours
0
2
4
6
8
10
12
14
TIME (hours)

a poor yield of vesicles resulting from the process of differential centrifugation. It should be recalled that only one-half a heart was used to obtain granules.

(2) In terms of catecholamine turnover in the heart (employing the perchlorate preparation), these results coincide closely with those obtained by other workers using similar methods. This lends some credibility to the validity of the experimental procedure.

(3) The lines of regression calculated from the kidney perchlorate preparation raise a number of important points. Before passage over alumina, the regression line does not fall within 2SE of the mean for two time points (3 and 7 hours), and barely within 2SE of the mean for the first time interval (one-half hour). Indeed, there appears to be a rapid fall off in ^{3}H-NE concentration within the first 3 hours, followed by a slower decline thereafter.

A two component line is suggested. The first component appears to include events from 0 to 3 hours; while the second component reflects subsequent turnover. The reason for this may be as follows: Within a short time after injection of ^{3}H-NE, unincorporated catecholamine as well as labeled O-methylated catabolites collect in the urine. When rats are sacrificed one-half hour post-injection, and the kidneys homogenized in perchlorate, much of this contamination has not yet been excreted. This contributes to radioactivity measured by scintillation spectrometry. However, within 3 hours following ^{3}H-NE administration, most of these catabolites and unincorporated ^{3}H-NE have been excreted.

Purification with alumina eliminates radioactive counts not emanating from ^{3}H-NE. The apparent concentration of incorporated ^{3}H-NE thus falls at time = one-half hour. And the only variable which may spuriously

elevate this quantity is unincorporated ^{3}H-NE. Support for this argument is implied by the line of regression determined after alumina. Here, the line falls within 2SE's of the mean for each time point.

The above observations suggest two recommendations for future turnover experiments using the kidney perchlorate preparation. First, the regression line should be calculated only from data obtained after passage over alumina. Second, turnover should be measured beginning 2 to 3 hours following iv. injection of ^{3}H-NE.

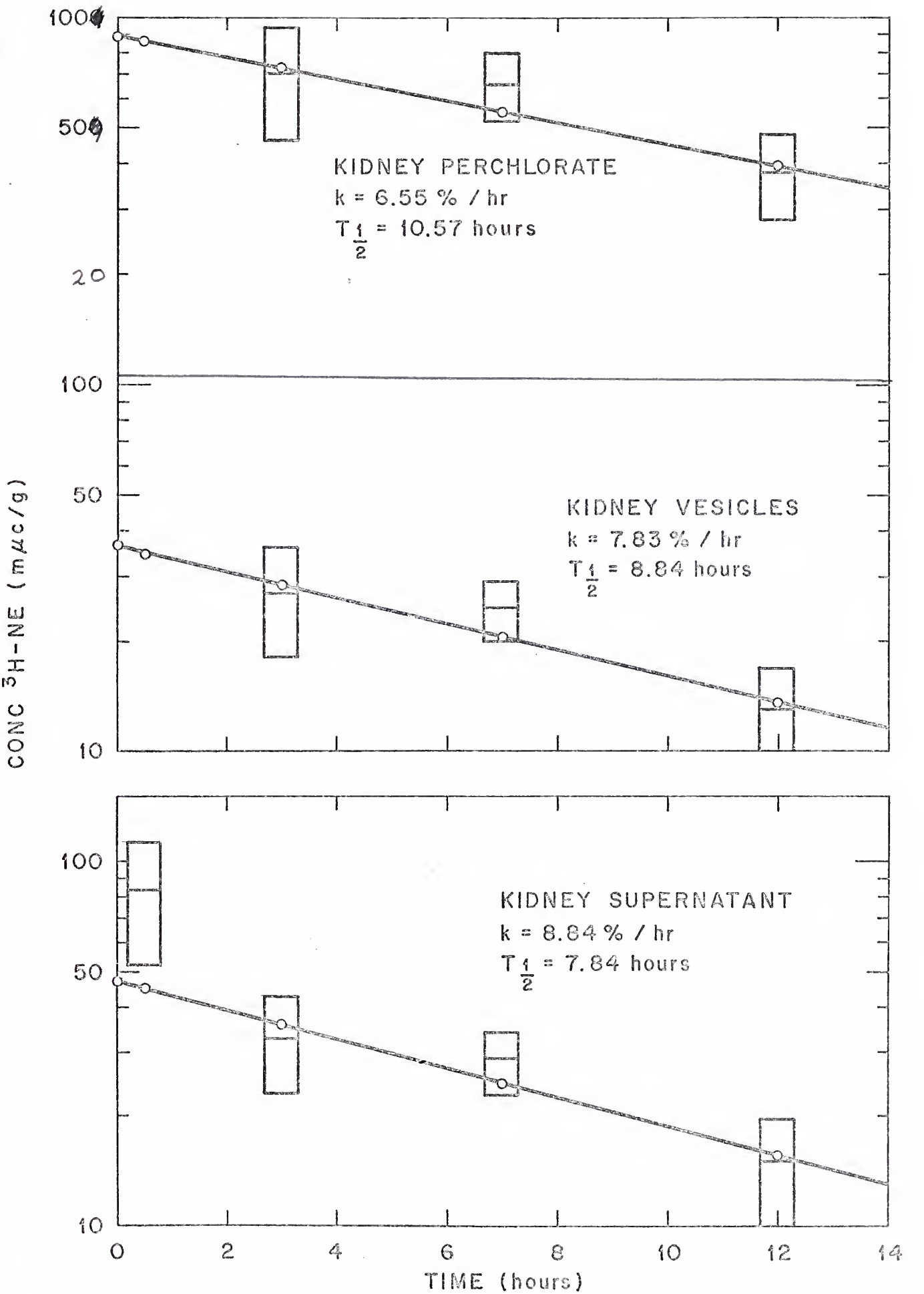
Figure 8 represents a recalculation of the line of regression for the kidney perchlorate preparation, after alumina, based on time points 3, 7, and 12 hours. The fractional turnover, turnover time, half-life, and coefficient of correlation are 6.55 per cent/hour, 15.26 hours, 10.57 hours, and 0.65, respectively.

(4) Regression lines for turnover in the kidney vesicles and supernatant may also be broken down into two components. Rapid fall off in concentration of ^{3}H-NE appears to occur in the first 3 hours, followed by a slower decline. However, in the vesicular preparation, the first component does not represent as fast a decline in ^{3}H-NE as its perchlorate analog. Nonetheless, the same explanation of the phenomenon used for the perchlorate preparation pertains here.

In Figure 8 are the lines of regression for turnover in vesicles and supernatant, respectively, based on ^{3}H-NE concentration at times 3, 7, and 12 hours (after alumina). Fractional turnover, turnover time, half-life, and coefficient of correlation for these lines are summarized in Table 21. In future turnover experiments using a vesicular preparation, the same recommendations apply as those outlined in (3) above.

(5) There appears to be no stastical difference between the lines

Fig. 8


KIDNEY PERCHLORATE
k = 6.55 % / hr
T 1/2 = 10.57 hours
KIDNEY VESICLES
k = 7.83 % / hr
T 1/2 = 8.84 hours
KIDNEY SUPERNATANT
k = 8.84 % / hr
T 1/2 = 7.84 hours
CONC 3H-NE (mμc/g)
TIME (hours)
100
50
20
10
0
2
4
6
8
10
12
14

Preparation	Fractional Turnover*	Turnover Time (hrs)	Half-life ^{3}H-NE (hrs)	Coefficient of Correlation
Kidney perchlorate (K)	6.55	15.26	10.57	- 0.6555
Kidney supernatant (K_s)	8.84	11.30	7.84	- 0.7542
Kidney vesicles (K_v)	7.83	12.77	8.85	- 0.7293

* % decline ^{3}H-NE (muc/g) per hour

Table 21. Kinetic parameters derived from regression lines recalculated for time: 3, 7, 12 hrs. (after passage over alumina)

Comparison of Regression Lines	t - value	p - value
Kidney vesicles (K_v) vs. Kidney perchlorate (K)	0.474	$p < 0.70$
Kidney vesicles (K_v) vs. Kidney supernatant (K_s)	0.360	$p < 0.80$
Kidney perchlorate (K) vs. Kidney supernatent (K_s)	0.803	$p < 0.50$

Table 22. Comparison of slopes of regression lines recalculated for time: 3, 7, and 12 hrs. (after passage over alumina).

of regression (after alumina) for turnover in the kidney vesicular and perchlorate preparations. This was revealed by determining the standard error of estimate about each line, and using this to compare slopes by the standard t-test. Table 22. Hence, it may be argued that one need not prepare vesicles via the arduous procedure of differential centrifugation in order to measure turnover of catecholamine. Instead, the perchlorate preparations are adequate in this regard, after passage over alumina.

VI. Effect of ganglionic blockage on catecholamine turnover

During periods of sympathetic ganglionic blockage, one would anticipate less adrenergic activity in end-organs. Consequently, there should be less turnover of catecholamine in sympathetic nerve endings. Under these conditions, ^{3}H-NE which is taken up by sympathetic nerves, and stored in vesicles, should enjoy a longer half-life than it does under normal circumstances. In this experiment (see Methods) chlorisondamine hydrochloride (Ecolid) was employed as the ganglionic blocking agent. It is of interest that those animals which received Ecolid demonstrated, quite dramatically, the extra-renal manifestations of ganglionic blockade: extreme ptosis and bladder distension.

A. Table 23 illustrates the pertinent data relative to the experimental set-up.

B. Concentration of ^{3}H-NE, 6 hours after iv. administration of this isotope, is presented for chlorisondamine-treated and control animals. Table 24.

C. For each preparation, the mean ($\bar{x}$), standard deviation (SD), and standard error (SE) were determined for ^{3}H-NE concentration, Table 25, (after alumina). These results are graphically portrayed in Figure 9.

D. Conclusions: That chlorisondamine significantly retards the turnover of catecholamine is readily apparent in Figure 9. This is confirmed by comparing control and chlorisondamine-treated groups by standard t-test, Table 26. Pharmacologic evidence has thus been adduced in support of the conclusion derived from the previous turnover experiment: that turnover of catecholamine does exist in the kidney.

Rat #	Wt. of rat(g)	Wt. of kidneys (g)	Wt. of heart (g)
1	215	1.46	0.62
2	202	1.52	0.60
3	200	1.42	0.62
4	190	1.45	0.56
5	187	1.36	0.52
6	214	1.84	0.70
7	195	1.63	0.54
8	202	1.60	0.62
9	192	1.54	0.57
10	204	1.50	0.60
11	205	1.39	0.55
12	213	1.98	0.65

Table 23.

	Rat#	Before Alumina Heart H	Kidney K_v	Kidney K_s	After Alumina Heart H	Kidney K_v	Kidney K_s
Control	2	2799	37	86	4722	40	47
	4	2642	35	85	4892	38	42
	6	3597	57	103	5970	67	58
	8	2853	43	97	5054	42	42
	10	2567	51	87	3515	58	54
	12	2566	39	79	4146	50	39
Ecolid	1	3640	101	111	5983	94	83
	3	4130	111	132	5802	122	109
	5	3365	97	135	5717	92	115
	7	3833	124	134	7264	71	112
	9	3958	104	104	7197	77	88
	11	4712	72	101	7182	70	74

H : heart perchlorate preparation; muc ^{3}H-NE/g
K_v : kidney vesicular preparation; muc ^{3}H-NE/g (Fraction V)
K_s : kidney supernatant preparation; muc ^{3}H-NE /g (Fraction IV)

Table 24.

	Preparation	$\bar{x}$	SD	SE	$\bar{x}$ + 2SE	$\bar{x}$ - 2SE
Control	Heart	4716	834	342	5400	4032
	K_v *	49	11	4	57	41
	K_s **	47	7	3	53	41
Ecolid	Heart	6524	761	312	7148	5900
	K_v	87	19	8	103	71
	K_s	96	17	7	110	82

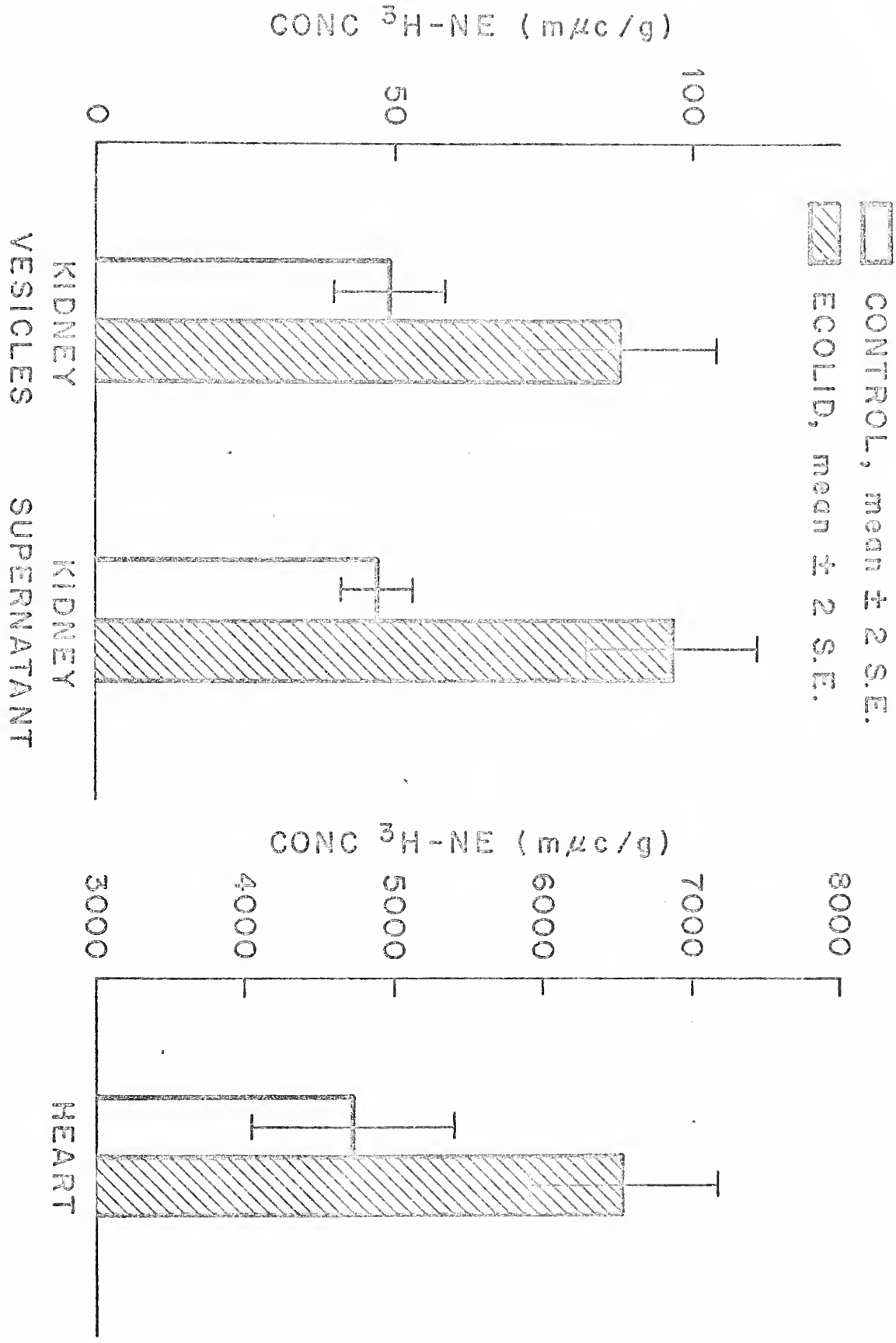
* : Kidney vesicles (Fr V)
** : Kidney supernatent (Fr IV)
Values are expressed in terms of muc/g ^{3}H-NE

Table 25.

Preparation	t-value	p-value
Heart	4.04	$p < 0.005$
Kidney vesicles	4.25	$p < 0.005$
Kidney supernatant	6.67	$p < 0.001$

Table 26.

Fig. 9


CONC ^{3}H-NE (mμc/g)
0
50
100
KIDNEY
VESICLES
KIDNEY
SUPERNATANT
CONTROL, mean ± 2 S.E.
ECOLID, mean ± 2 S.E.
CONC ^{3}H-NE (mμc/g)
3000
4000
5000
6000
7000
8000
HEART

DISCUSSION

The experimental results presented in this paper support the contention that catecholamine turnover in the kidney exists and can be measured. Methods employed in obtaining this information were predicated upon the principles of steady state kinetics. Conclusions derived from this analysis were corroborated by the demonstration that sympathetic ganglionic blockade significantly retards catecholamine turnover.

In the conduct of this research, however, a few problems were encountered which have some bearing on the interpretation of the experimental results. For example, the endogenous assay of NE, as described by von Euler and Lishajake, was frustrated by the presence of a nonproteinaceous contaminant in the kidney. This was not present in rat heart or urine. Addition of a Dowex cation exchange column to the standard method alumina chromatography did not prove of value in overcoming this problem. Hence, adrenergic activity was quantified in terms of mμc ^{3}H-NE per gram of kidney tissue. The assumptions upon which this unit of measurement is based are elaborated in Materials and Methods. Clearly, this is not as significant a measurement of sympathetic tone as specific activity would have been (mμc ^{3}H-NE/ng NE). In future experiments, one may approach this problem by determining endogenous NE via a recently developed enzyme radiochemical assay for NE.

Another problem which defied complete resolution was an inability to inhibit the small amount of ^{3}H-NE which is adsorbed by (or taken up by) the neuronal vesicles. This phenomenon has been described for other populations of vesicles and is temperature-dependent. Though unable to totally prevent adsorption, the process was minimized in these experiments by maintaining the vesicular preparations at 4°C. It should also be mentioned that in the course of investigating this problem, EDTA was found

to either destroy or dramatically alter the sedimentation properties of rat heart and kidney granules.

One additional criticism may be leveled against the turnover experiments performed here. Only five rats comprised each time interval. To have used more animals would have proved exceedingly difficult, given the facilities available to the author for isolating granules. Ideally, one would require far more than this number in order to minimize the standard error which derives from the biological variation of such a small group.

A finding of significant relevance to this problem is the lack of statistical difference between the regression lines obtained from kidney vesicular and perchlorate preparations (after passage over alumina, and calculated for time 3, 7, 12 hours). The implication of this is obvious: turnover in the kidney may be measured using the perchlorate perparation, as is done with the heart. Thus, the need to isolate vesicles by the arduous procedure of differential centrifugation is obviated. One could then easily include ten or more rats per time interval, and thereby improve the statistical significance of the results.

What other experiments might be conducted to lend further support for the conclusions reached from this research? One exciting possibility involves the use of 6-hydroxydopamine. In high doses, this agent produces degenerative lesions in terminal adrenergic fibers, comparable to those produced after surgical denervation (74). Within 1-2 hours following intravenous injection of 6-hydroxydopamine (100 mg/kg) into rats, endogenous NE of the heart declines 70-90 per cent. This catecholamine depletion persists for weeks, and slowly returns thereafter, along with regeneration of the adrenergic terminal fiber plexus.

Using 6-hydroxydopamine, one could set-up a study designed to

demonstrate that iv. administered ^{3}H-NE is incorporated into the sympathetic neuronal granules of the kidney. Just prior to administration of ^{3}H-NE, experimental animals would be given 6-hydroxydopamine iv. (100 mg/kg). Controls would receive saline. Six to eight hours following injection of ^{3}H-NE, animals would be sacrificed and kidney and heart perchlorate preparations obtained. One would expect to see a much diminished concentration of ^{3}H-NE in tissue from rats treated with 6-hydroxydopamine, compared with controls.

Such experiments have already been conducted in the laboratory of Dr. Lewis Landsberg at Boston City Hospital (personal communication). His results are in accordance with the expectations discussed above.

Having established that renal catecholamine turnover exists and can be quantified, researchers may direct their efforts toward correlating sympathetic activity with changes in sodium metabolism, using the methods worked out here. These investigations could provide much needed insight into the pathophysiology of hypertension and edema-forming states.

BIBLIOGRAPHY

1. Earley, L.E., Daugharty, T.M.: Sodium metabolism. New Eng. J. Med. 281:72-86, 1969.

2. DeWardner, H.E., Mills, I.H., Clapham, W.F., Hayter, C.J.: Studies of efferent mechanism of sodium diuresis which follows administration of intravenous saline in the dog. Clin. Sci. 21:249-258, 1961.

3. Martino, J.A., Earley, L.E.: Demonstration of the role of physical factors as determinants of natriuretic response to volume expansion. J. Clin. Invest. 46:1963-1978, 1967.

4. Rector, F.C.: Demonstration of hormonal inhibitor of proximal tubular reabsorption during expansion of extracellular volume with istonic saline. J. Clin. Invest. 47:761-773, 1968.

5. Giebisch, G.: Coupled ion and fluid transport in the kidney. New Eng. J. Med. 287:913-919, 1972.

6. Nilsson, O.: The adrenergic innervation of the kidney. Lab. Invest. 14:1392-1395, 1965.

7. Christensen, K., Lewis, E.L., Kuntz, A.: Innervation of the renal blood vessels in the cat. J. Comp. Neurol. 95:373-385, 1951.

8. McKenna, O.C., Angelakos, E.T.: Adrenergic innervation of the canine kidney. Circ. Res. 22:345-353, 1968.

9. Ljungqvist, A., Wagermark, J.: The adrenergic innervation of intrarenal glomerular and extraglomerular circulatory routes. Nephron. 7:218-229, 1970.

10. Zimmerman, B.G., Liao, J.C., Gisslen, J.: Effects of phenoxybenzamine and combined administration of ipromiazid and tropolone on catecholamine release elicited by renal sympathetic nerve stimulation. J. Pharmacol. Exp. Ther. 176:603-610, 1971.

11. Coucha, J., Norris, B.: Studies on renal vasomotion. Brit. J. Pharmacol. 34:277-290, 1968.

12. Iriuchijima, J., Wilson, M.F.: Sympathetic vasoconstrictor activity to the kidney in carotid occlusion pressor reflex. Proc. Soc. Exptl. Biol. Med. 131:189-192, 1969.

13. Ninomiya, I., Irisawa, K.: Summation of baroreceptor reflex effects on sympathetic nerve activity. Am. J. Physiol. 216:1330-1336, 1969.

14. Wilson, M.F., Ninomiya, I., Franz, G.N., Judy, W.V.: Hypothalamic stimulation and baroreceptor reflex interaction on renal nerve activity. Am. J. Physiol. 221:1768-1773, 1971.

15. Gill, J.R., Casper, A.G.T.: Role of the sympathetic nervous system in the renal response to hemorrhage. J. Clin. Invest. 48:915-922, 1969.

16. Gill, J.R., Barther, F.C.: Adrenergic nervous system in sodium metabolism. II. Effects of guanethidine on the renal response to sodium deprivation in normal man. New Eng. J. Med. 275:1466-1470, 1966.

17. Shear, L.: Renal function and sodium metabolism in idiopathic orthostatic hypotension. New Eng. J. Med. 268:347-352, 1963.

18. Gill, J.R., Mason, D.T., Barther, F.C.: Adrenergic nervous system in sodium metabolism: Effects of guanethidine and sodium-retaining steroids for normal man. J. Clin. Invest. 43:177-184, 1964.

19. Truta, J., Barclay, A.E., Daniel, D.M., Franlin, K.J., Pritchard, M.M.L.: Studies of the Renal Circulation. Charles C. Thomas, Ed., Springfield, Ill., 1947.

20. Pomeranz, B.H., Birtch, A.G., Barger, A.C.: Neural control of intrarenal blood flow. Am. J. Physiol. 215:1067-1081, 1968.

21. Carriere, S., Thorburn, G.D., O'Marchoe, C.C.C., Barger, A.C.: Intrarenal distribution of blood flow in dogs during hemorrhage hypotension.

Circ. Res. 19:167-179, 1966.

22. Horster, M., Thueran, K.: Micropuncture studies on the filtration rate of single superficial and juxtamedullary glomeruli in the rat kidney. Arch. Ges. Physiol. 301:162-181, 1968.

23. Hollenberg, N.K., Epstein, M., Guttmann, R.D., Conray, M., Basch, R.I., Merrill, J.P.: Effect of sodium balance on intrarenal distribution of blood flow in normal man. J. Appl. Physiol. 28:312-317, 1970.

24. Wogermark. J., Ungerstedt, U., Ljungqvist, A.: Sympathetic innervation of the juxtaglomerular cells of the kidney. Circ. Res. 22:149-153, 1968.

25. Barajas, L., Lotta, H.: A three-dimensional study of the justoglomerular apparatus in the rat. Lab. Invest. 12:257-269, 1963.

26. Barajas, L.: The innervation of the juxtoglomerular apparatus. Lab. Invest. 13:916-929, 1964.

27. Vander, A.J.: Effect of catecholamines and the renal nerves on renin secretion in anesthetized dogs. Am. J. Physiol. 209:659-662, 1965.

28. Taquini, A.C., Blaquier, P.: On the production and role of renin. Canad. Med. Assoc. J. 90:210, 1963.

29. Bunag, R.D., Page, I.H., McCubbin, J.W.: Neural stimulation of release of renin. Circ. Res. 19:851-858, 1966.

30. Mogil, R.A., Itshovitz, H.D., Russell, J.H., Murphy, J.J.: Renal innervation and renin activity in salt metabolism and hypotension. Am. J. Physiol. 216:693-697, 1969.

31. Vander, A.J.: Control of renin release. Physiol. Rev. 47:359-382, 1967.

32. Gordon, R.D., Kuchel, O., Liddle, G.W., Island, D.P.: Role of the sympathetic nervous system in regulating renin and aldosterone production in man. J. Clin. Invest. 46:599-605, 1967.

33. Ueda, H., et al.: Observations on the mechanism of renin release by hydalazine in hypertensive patients. Circ. Res. (suppl. II) 27:201-206, 1970.

34. Winer, N., Cholski, D.S., Scen Yoon, M., Freedman, A.D.: Adrenergic receptor mediation of renin secretion. J. Clin. Endocr. Metab. 29:1168-1175, 1969.

35. Oliver, W.J., Lelsch, R.C., Chandler, J.P.: Demonstration of increased catecholamine excretion in the nephrotic syndrome. Proc. Soc. Exp. Biol. 125:1175-1180, 1967.

36. Chidsey, C.A., Braunwald, E., Marrow, A.G.: Catecholamine excretion and cardiac stores of norepinephrine in congestive heart failure. Am. J. Med. 39:442-451, 1965.

37. Chidsey, C.A., Hanison, D.C., Braunwald, E.: Augmentation of plasma norepinephrine response to exercise in patients with congestive heart failure. New Eng. J. Med. 267:650-654, 1962.

38. Braunwald, E., Chidsey, C.A., Pool, P.E., Sonneblick, E.N., Ross, J., Covell, J.W.: Congestive heart failure: biochemical and physiological considerations. Ann. Intern. Med. 64:904-941, 1966.

39. Gill, J.R.: The Role of the Sympathetic Nervous System in the Regulation of Sodium Excretion by the Kidney. From Frontiers in Neuroendocrinology, 1969. Ed. by W.F.Ganong. Oxford Univ. Press.

40. Kilcayne, M.M., Cannon, P.J.: Influence of thoracic caval occlusion on intrarenal blood flow distribution and sodium excretion. Am. J. Physiol. 220:1220-1230, 1971.

41. Kilcayne, M.M., Cannon, P.J.: Neural and humoral influences on intrarenal blood flow distribution during thoracic inferior vena caval occlusion. Am. J. Physiol. 220:1231-1237, 1971.

42. Sparks, H.V., Kopald, H.H., Carriere, S., Chimoskey, J.E., Barger, A.C.: Intrarenal distribution of blood flow in dogs with chronic congestive heart failure. Physiologist 8:277, 1965.

43. Kinstiner, N.: Uptake of catecholamines by a particulate fraction of the adrenal medulla. J. Biol. Chem. 237:2311-2317, 1962.

44. Wolf, D.E., Potter, L.T., Richardson, K.C., Axelrod, J.: Localizing tritiated norepinephrine in sympathetic axons by electron microscope autoradiography. Science 138:440, 1962.

45. Potter, L.T., Axelrod, J.: Subcellular localization of catecholamines in tissues of the rat. J. Pharmacol. Exp. Ther. 142:291-298, 1963.

46. Geffen, L.B., Livett, B.G.: Synoptic vesicles in sympathetic neurons. Physiol. Rev. 51:98-157 , 1971.

47. Landsberg, L., Levine, R., Catecholamines and the adrenal medulla in Duncan's Diseases of Metabolism, Philip K. Bondy, M.D., Ed., 7th Edition (in press).

48. Undenfriend, S., Zoltzman-Nirenberg, P., Gordon, R., Spector, S.: Evaluation of the biochemical effects produced in vivo by inhibitors of the three enzymes involved in norepinephrine biosynthesis. Mol. Pharmacol. 2:95-105, 1966.

49. Nogatsen, T., Rust, L.A., DeQuattro, V.: The activity of tyrosine hydroxylase and related enzymes of catecholamine biosynthesis and metabolism in dog kidney--effects of denervation. Biochem. Pharmacol. 18:1441-1446, 1969.

50. Titus, E., Dengler, H.J.: The mechanism of uptake of norepinephrine. Pharmacol. Rev. 18:525-535, 1966.

51. Michaelson, I.A., Richardson, K.C., Snyder, S.N., Titus, E.O.: The separation of catecholamine storage vesicles from rat heart. Life Sci. 3:971-978, 1964.

52. Potter, L.T.: Storage of norepinephrine in sympathetic nerves. Pharmacol. Rev. 18:439-451, 1966.

53. Stjarne, L.: Studies of catecholamine uptake, storage and release mechanisms. Acta Physiol. Scand. 62 (suppl.):228, 1964.

54. Potter, L.T.: Role of intraneuronal vesicles in the synthesis, storage and release of noradrenalin. Circ. Res. (Suppl. III to Vols. 20,21: III - 13 - III - 24), 1967.

55. Schneider, F.H., Smith, A.D., Winkler, H.: Secretion from the adrenal medulla: biochemical evidence of exocytosis. Brit. J. Pharmacol. Chemother. 31:94-104, 1967.

56. Douglas, W.W.: The mechanism of release of catecholamines from the adrenal medulla. Pharmacol. Rev. 18:471-480, 1966.

57. Viveros, O.H., Arqueros, L., Kirshner, N.: Release of catecholamines and dopamine-B-oxidase from the adrenal medulla. Life Sci. 7:609-618, 1968.

58. DePotter, W.P., DeSchaepdryer, H., Moerman, E., Smith, A.D.: Evidence for the release of vesicle proteins together with noradrenalin upon stimulation of the splenic nerves. J. Physiol. (London) 204:102^{P}-104^{P}, 1969.

59. Geffer, L.B., Livett, B.G., Rush, R.A.: Immunological localization of chromogravins in sheep sympathetic neurons and their release by nerve impulses. J. Physiol. (London) 204:58-59^{P}, 1969.

60. Hertting, G., Axelrod, J., Kopin, I.J., Whitby, L.G.: Lack of uptake of catecholamines after chronic denervation of sympathetic nerves. Nature 189:66, 1961.

61. Whitby, L.G., Axelrod, J., Weil-Malherbe, H.: Fate of ^{3}H-NE in animals. J. Pharmacol. Exp. Ther. 132:193-201, 1961.

62. Montonari, R., Costa, E., Beavin, M.A., Brodie, B.B.: Turnover rates of norepinephrine in hearts of intact mice, rats and guinea pig using tritiated norepinephrine. Life Sci. 2:232-240, 1963.

63. Costa, E., Boulin, D.J., Hammer, W., Vogel, W., Brodie, B.B.: Interaction of drugs with adrenergic neurons. Pharmacol. Rev. 18:577-597, 1966.

64. Neff, N.H., Tozer, T.N., Hammer, W., Costa, E., Brodie, B.B.: Application of steady-state kinetics to the uptake and decline of ^{3}H-NE in the rat heart. J. Pharmacol. Exp. Ther. 160:48-52, 1968.

65. Landsberg, L., DeChamplain, J., Axelrod, J.: Increased biosyntheses of cardiac norepinephrine hypophysectomy. J. Pharmacol. Exp. Ther. 165:102-107, 1969.

66. Rubenson, A.: Alterations in norepinephrine turnover in the peripheral sympathetic neurons induced by stress. J. Pharm. Pharmacol. 21:878-880, 1969.

67. Taubin, H.L., Djahanguiri, B., Landsberg, L.: Norepinephrine concentration turnover in different regions of the gastrointestinal tract of the rat; an approach to the evaluation of sympathetic activity in the gut. Gut 13:790-795, 1972.

68. Brodie, B.B., Costa, E., Dlabac, A., Neff, N.H., Smookler, H.H.: Application of steady-state kinetics to the estimation of synthesis rate and turnover time of tissue catecholamines. J. Pharmacol. Exp. Ther. 154:493-498, 1966.

69. Kopin, I.J.: Biochemical aspects of release of norepinephrine and other amines from sympathetic nerve endings. Pharmacol. Rev. 18:513-523, 1966.

70. Anton, A.H., Sayre, D.F.: Study of the factors affecting the aluminum-oxide-trihydroxyindole procedure for the analysis of catecholamines. J. Pharmacol. Exp. Ther. 138:360-375, 1962.

71. DeChamplain, J., Krakoff, L.R., Axelrod, J.: Catecholamine metabolism in experimental hypertension in the rat. Circ. Res. 20:136-145, 1967.

72. Von Euler, U.S., Lishajko, F.: Improved technique for the fluorimetric estimation of catecholamine. Acta Physiol. Scand. 51:348-355, 1961.

73. Gillis, C.N.: Characteristics of norepinephrine retention by a subcellular fraction of rabbit heart. J. Pharmacol. Exp. Ther. 146: 54-60, 1964.

74. DeChamplain, J., Nadeau, R.: 6-Hydroxydopamine, 6-hydroxydopa and degeneration of adrenergic nerves. Fed. Proc. 30:877-885, 1971.

d

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS DATE